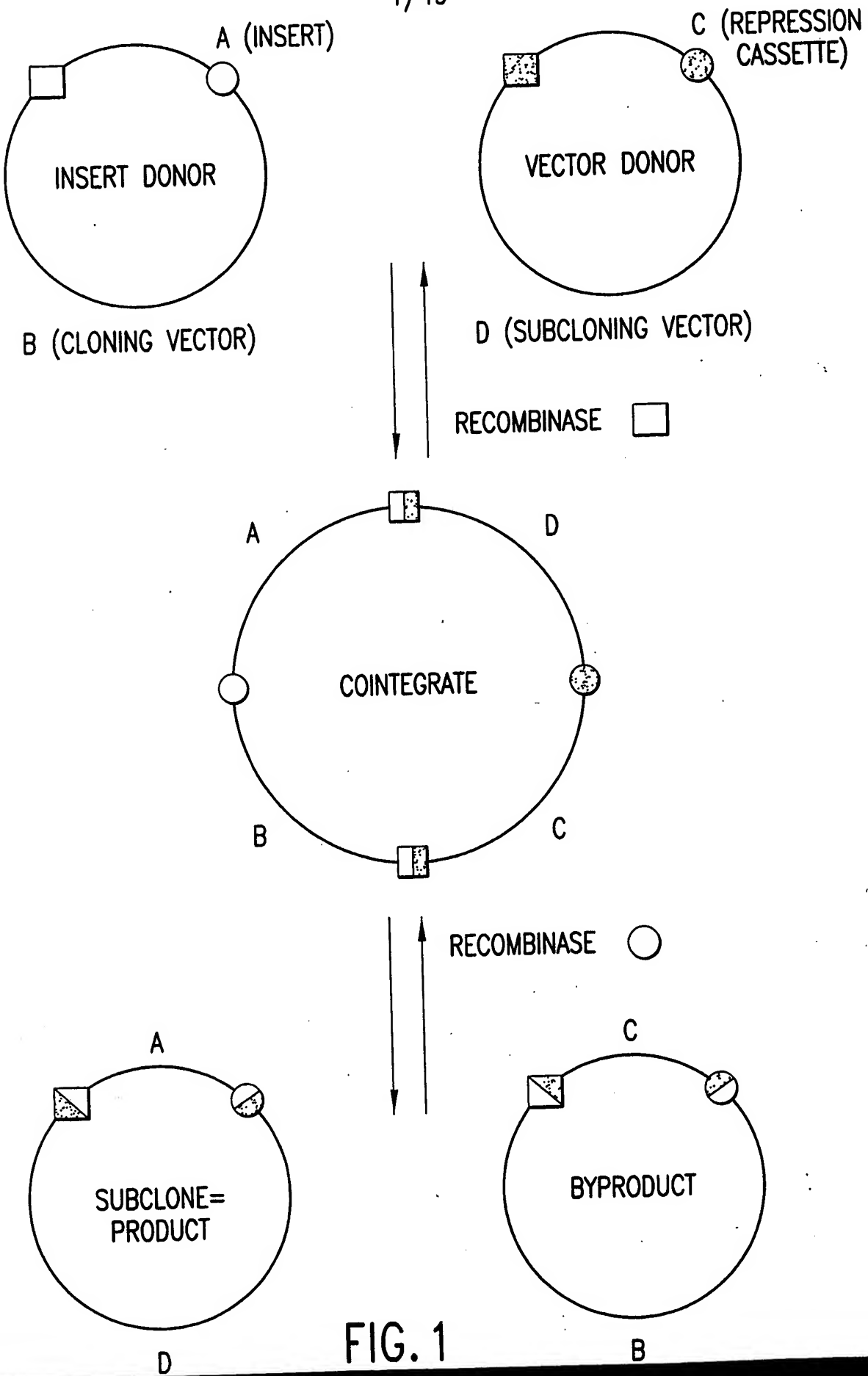
A (INSERT)
INSERT DONOR
B (CLONING VECTOR)
C (REPRESSION CASSETTE)
VECTOR DONOR
D (SUBCLONING VECTOR)
RECOMBINASE
A
D
COINTEGRATE
B
C
RECOMBINASE
A
SUBCLONE= PRODUCT
D
C
BYPRODUCT
B

FIG. 1

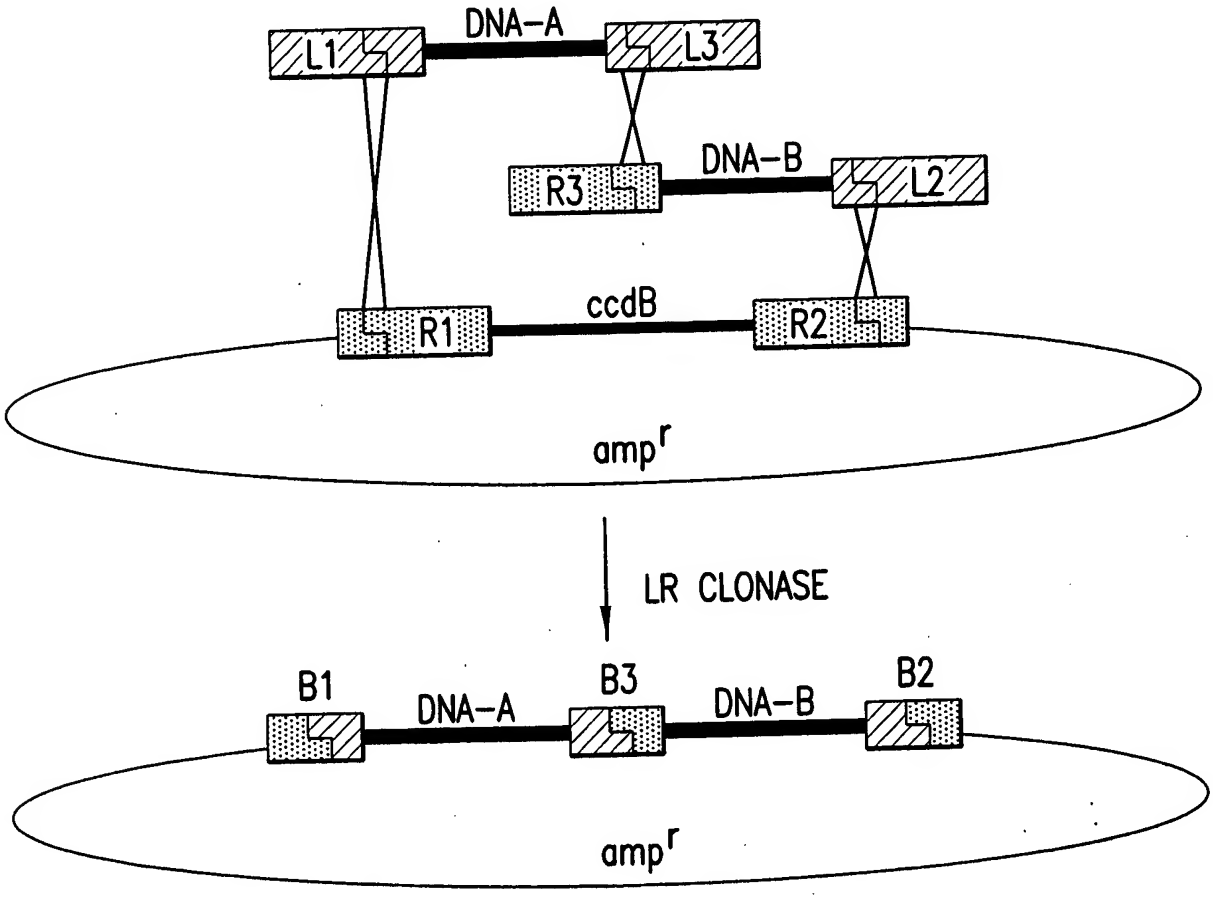
L1
DNA-A
L3
R3
DNA-B
L2
R1
ccdB
R2
amp^r
LR CLONASE
B1
DNA-A
B3
DNA-B
B2
amp^r

FIG. 2

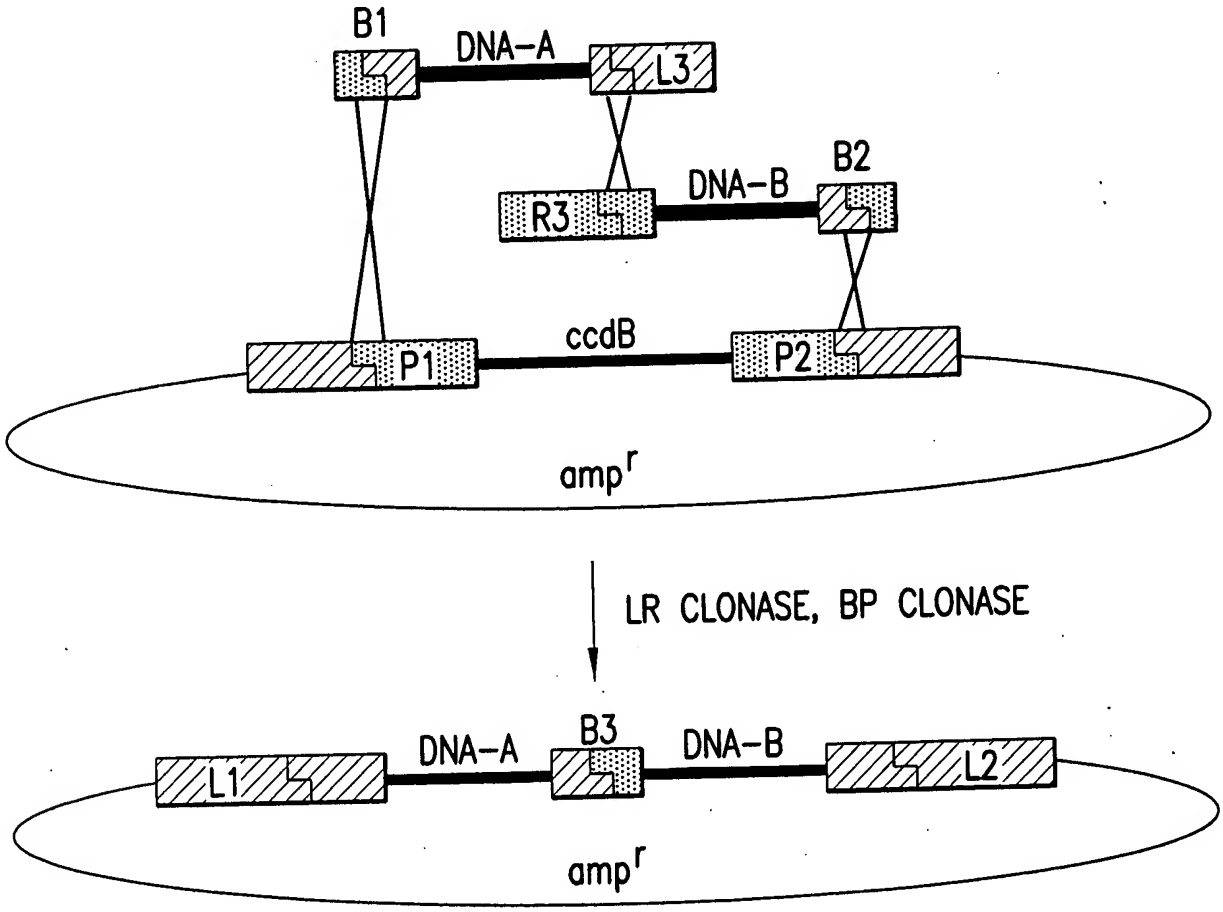
B1
DNA–A
L3
B2
DNA–B
R3
ccdB
P1
P2
amp^r
LR CLONASE, BP CLONASE
B3
DNA–A
DNA–B
L1
L2
amp^r

FIG. 3

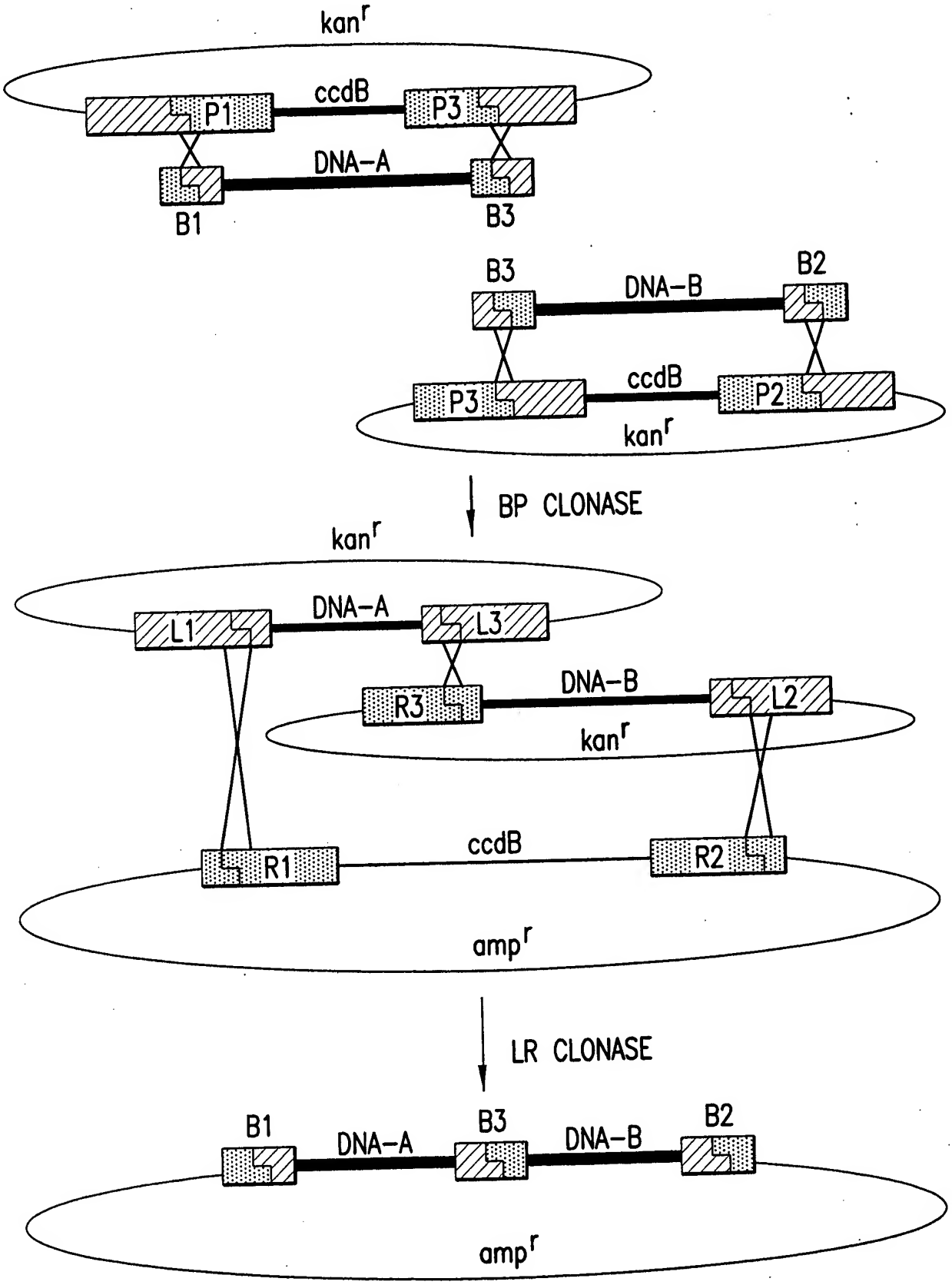
kan^r
P1
ccdB
P3
DNA–A
B1
B3
B3
DNA–B
B2
P3
ccdB
P2
kan^r
BP CLONASE
kan^r
L1
DNA–A
L3
R3
DNA–B
L2
kan^r
R1
ccdB
R2
amp^r
LR CLONASE
B1
DNA–A
B3
DNA–B
B2
amp^r

FIG. 4

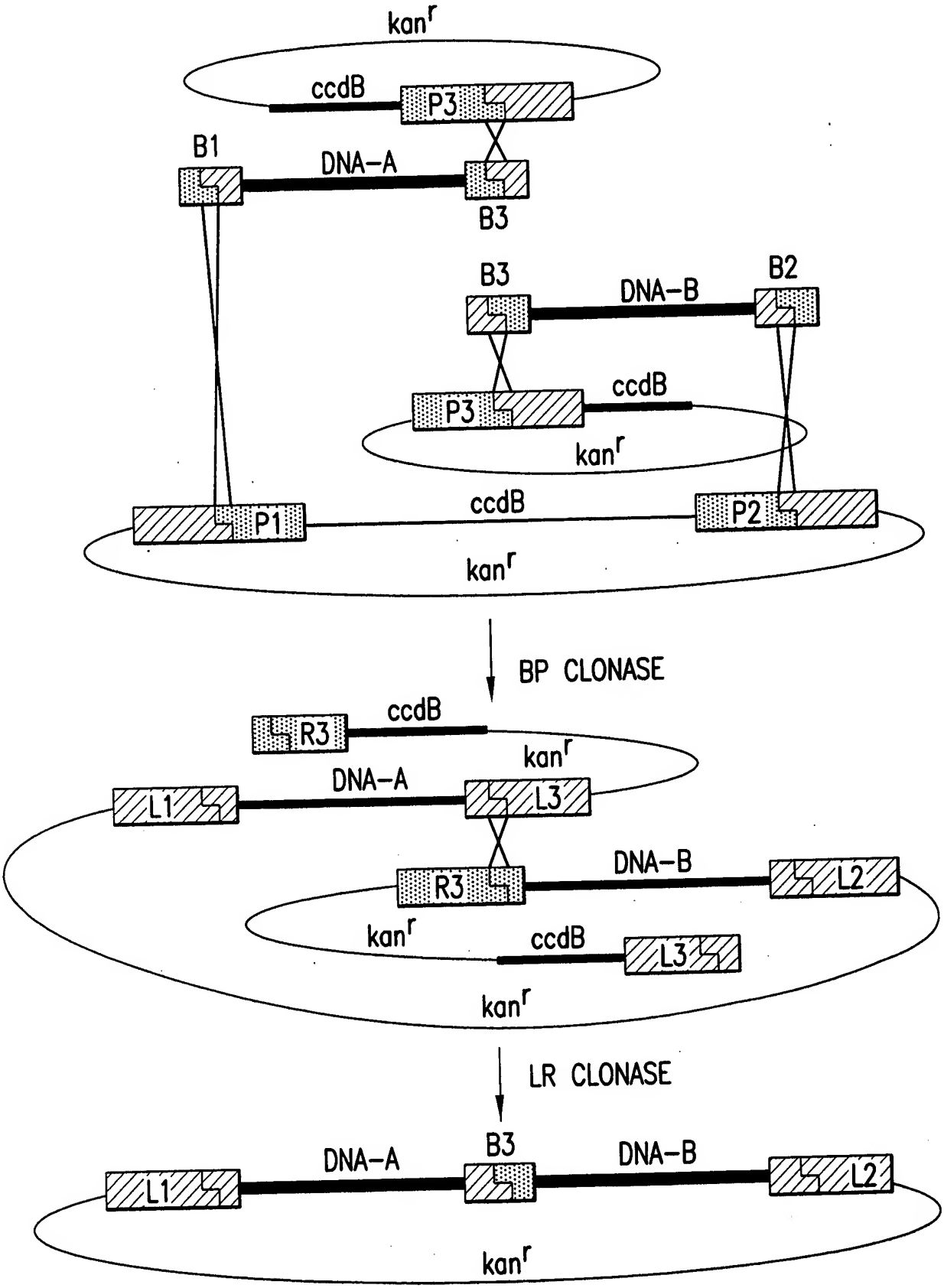
kan^r
ccdB
P3
B1
DNA–A
B3
B3
DNA–B
B2
ccdB
P3
kan^r
ccdB
P1
P2
kan^r
BP CLONASE
ccdB
R3
kan^r
DNA–A
L1
L3
DNA–B
R3
L2
kan^r
ccdB
L3
kan^r
LR CLONASE
DNA–A
B3
DNA–B
L1
L2
kan^r

FIG. 5

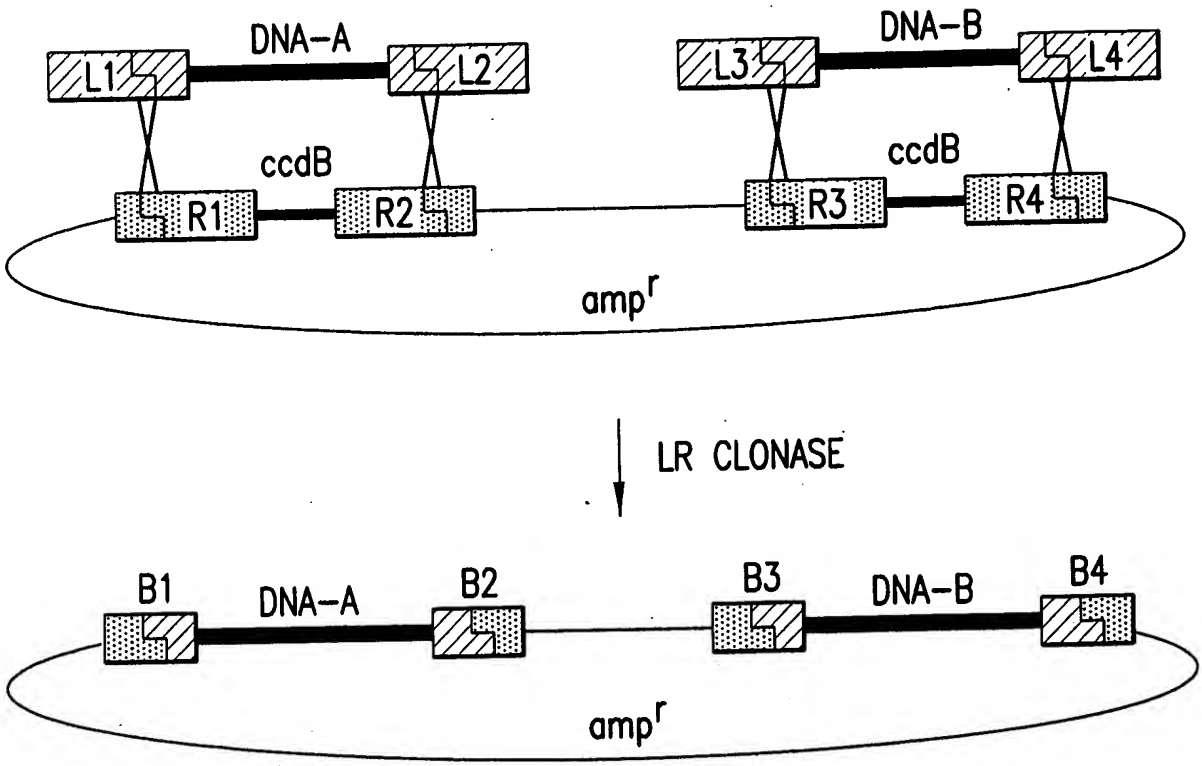
DNA–A
L1
L2
ccdB
R1
R2
DNA–B
L3
L4
ccdB
R3
R4
amp^r
LR CLONASE
B1
DNA–A
B2
B3
DNA–B
B4
amp^r

FIG. 6

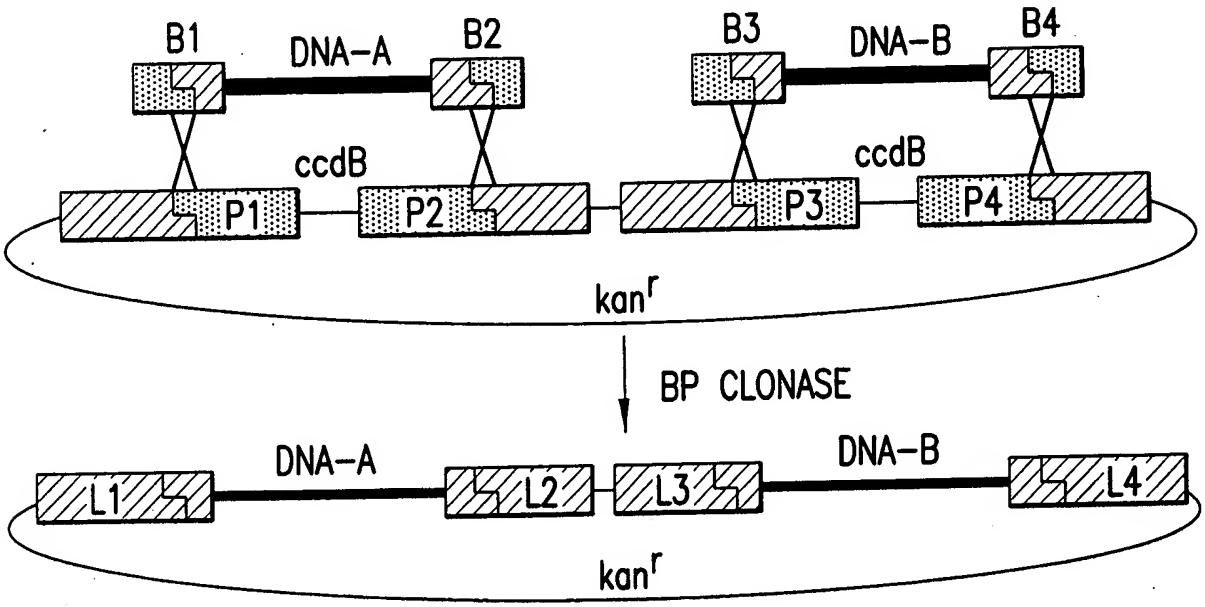
B1
DNA-A
B2
B3
DNA-B
B4
ccdB
ccdB
P1
P2
P3
P4
kan^r
BP CLONASE
DNA-A
DNA-B
L1
L2
L3
L4
kan^r

FIG. 7

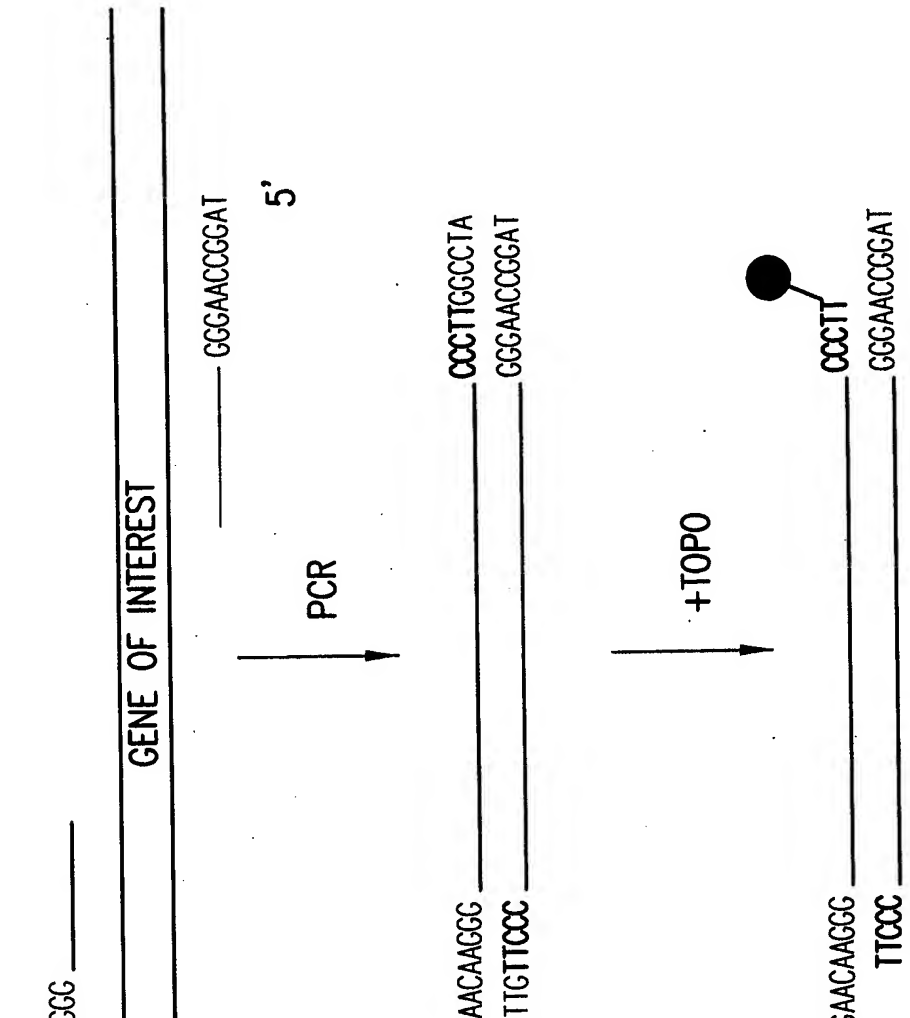
5'
CGGAACAAGGG
GENE OF INTEREST
GGGAACCGGAT
5'
PCR
CGGAACAAGGG
GCCTTGTTCCC
CCCTTGGCCTA
GGGAACCGGAT
+TOPO
CGGAACAAGGG
TTCCC
CCCTT
GGGAACCGGAT

FIG. 8A

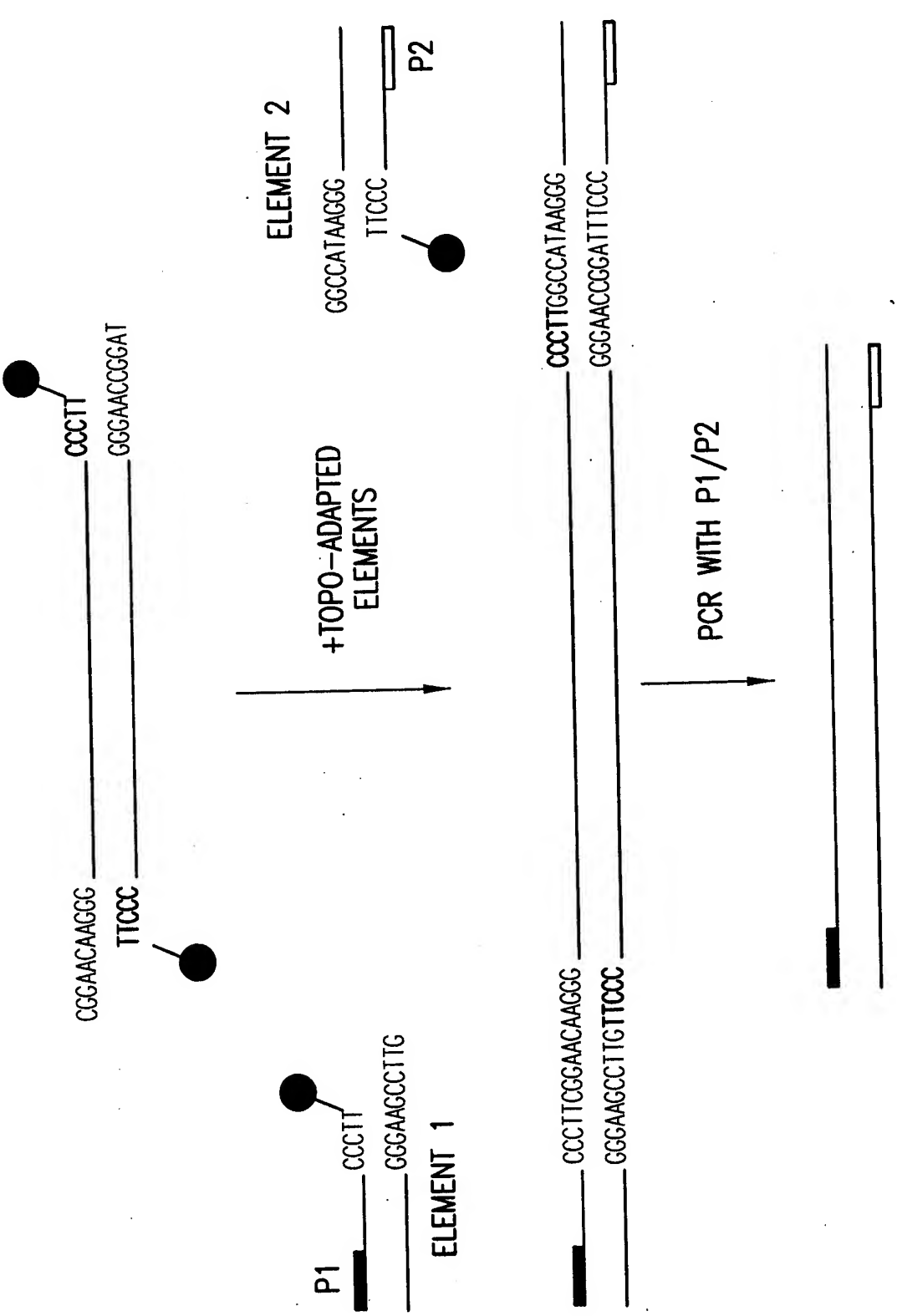
CCCTT
GGGAACCGGAT
CGGAACAAGGG
TTCCC
P1
CCCTT
GGGAAGCCTTG
ELEMENT 1
+TOPO-ADAPTED ELEMENTS
ELEMENT 2
GGCCATAAGGG
TTCCC
P2
CCCTTGGCCATAAGGG
GGGAACCGGATTTCCC
CCCTTCGGAACAAGGG
GGGAAGCCTTGTTCCC
PCR WITH P1/P2

FIG. 8B

Inventor(s): Chesnut *et al.*; Tel: 202/371-2600
Title: Method and Compositions for Synthesis of Nucleic Acid...

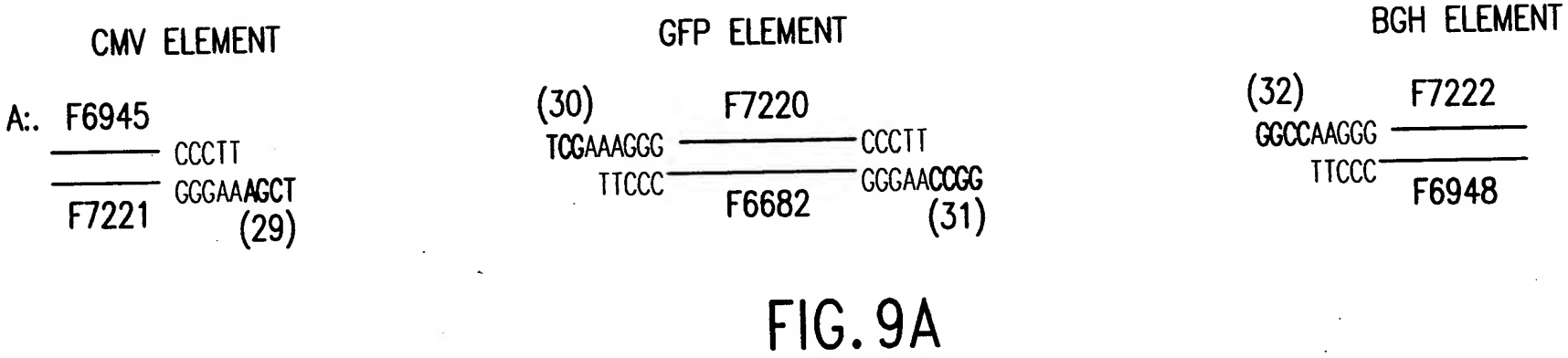

FIG. 9A

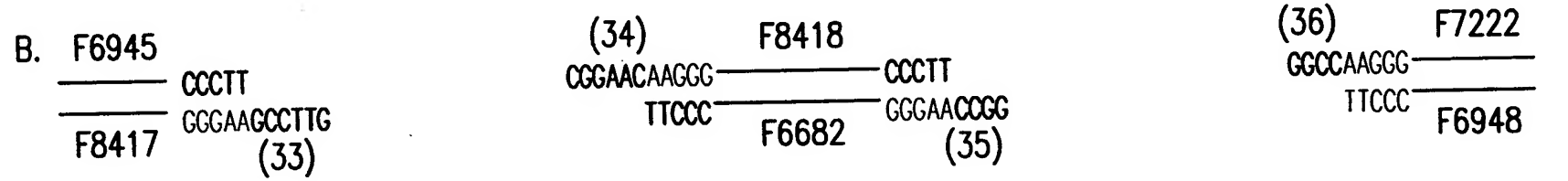

FIG. 9B

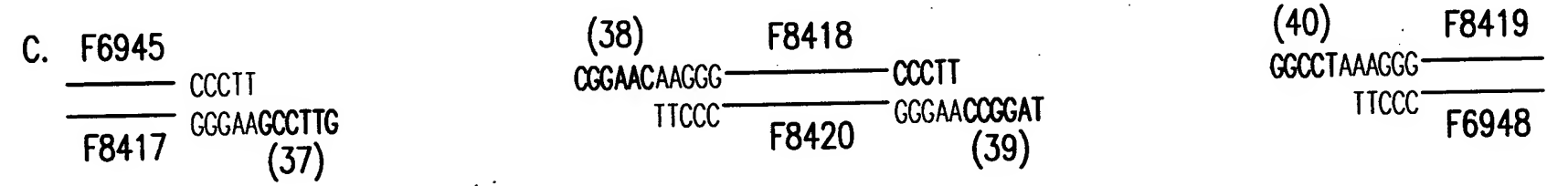

FIG. 9C

TABLE 1

Primer name	F#	Sequence (5'-> 3')	SEQ ID NO:
MTH1	10779	TATGTATCATACACATACGATTTAGGT	1
MTH2	10780	ACCGCCTCTCCCCGCGCGTT	2
GAL4r2	12667	GTTCCGAAGGGGGCGATACAGTCAACTGTCTTTG	3
MTH5	12505	TTGGCCAAGGGTATCTAGAAGCTTCTGCAGACGCGT	4
VP16r2	12668	GTTCCGAAGGGCCACCGTACTCGTCAATTCCAAG	5
SV40pAf	12016	GGCCAAAAGGGAACTTGTTTATTGCAGCTTATAATG	6
SV40pAr	561	CTCTGACTTGAGCGTCGATTTT	7
p53f2	12669	CGGAACAAGGGGAATTCCCTGTCACCGAGACC	8
SVTf2	12670	CGGAACAAGGGGAATTCCCGGGGATCTGGAATTC	9
CMVr2	7221	TCGAAAGGGTCGAGGTCGACCTGCAGCTG	10
CMVf	6945	AATTCACATTGATTATTGAGTAGTTA	11
GFP-Xhof	7220	TCGAAAGGGTAATGGCCAGCAAAGGAGAAG	12
GFP-Notr	6682	GGCCAAGGGTTTGTAGAGCTCATCCAT	13
BGHf2	7222	GGCCAAGGGTCTGAATGGGGCCGCATAGT	14
BGHr	6948	AAGCCATAGAGCCCGGGCCA	15
CMVr3	8417	GTTCCGAAGGGTCGAGGTCGACCTGCAGCTG	16
GFPf3	8418	CGGAACAAGGGATGGCCAGCAAAGGAGAAG	17
GFPr3	8420	TAGGCCAAGGGTTTGTAGAGCTCATCCATGC	18
BGHf3	8419	GGCCTAAAGGGTGAATGGGGCCGCATAGT	19
T7top	9304	GAAGGAGTAATACGACTCACTATAGGGAGCCACCATGGGCCCTTCGGAAC	20
T7bottom	9305	GTTCCGAAGGGCCCATGGTGGCTCCCTATAGTGAGTCGTATTACTCCTTC	21
T7amp	9306	GAAGGAGTAATACGACTCACT	22
T3top	9661	GGCCTAAAGGGTCCCTTTAGTGAGGGTTAATTGCGCGC	23
T3bottom	9662	GCGCGCAATTAACCCTCACTAAAGGGACCCTTTAGGCC	24
lacZf2	10632	CGGAACAAGGGATGATAGATCCCGTCGTTTTACA	25
lacZ1k2	10770	TAGGCCAAGGGGACCATTTTCAATCCGCACCT	26
lacZ2k2	10771	TAGGCCAAGGGGAGGCACTTCACCGCTTGCCA	27
lacZ3k2	10772	TAGGCCAAGGGTTTGACACCAGACCAACTGGTA	28

FIG. 9D

SAMPLE #	GAL4+pA	VP16+pA	pGene/lacZ	GAL4+p53+pA	VP16+T+pA	p53–VP16
1			0.26 μg	p 0.37 μg	p 0.37 μg	
2			0.4 μg	p 0.3 μg	p 0.3 μg	
3			0.4 μg			p 0.6 μg
4			0.4 μg	10.3 μg	10.3 μg	
5		10.3 μg	0.4 μg	10.3 μg		
6	10.3 μg		0.4 μg		10.3 μg	
7			0.4 μg	4.5 μl PCR	4.5 μl PCR	
8		4.5 μl PCR	0.4 μg	4.5 μl PCR		
9	4.5 μl PCR		0.4 μg		4.5 μl PCR	

FIG. 10A

SAMPLE #	LacZ activity
1	240000
2	140000
3	1800000
4	1400000
5	54000
6	80000
7	320000
8	12000
9	42000

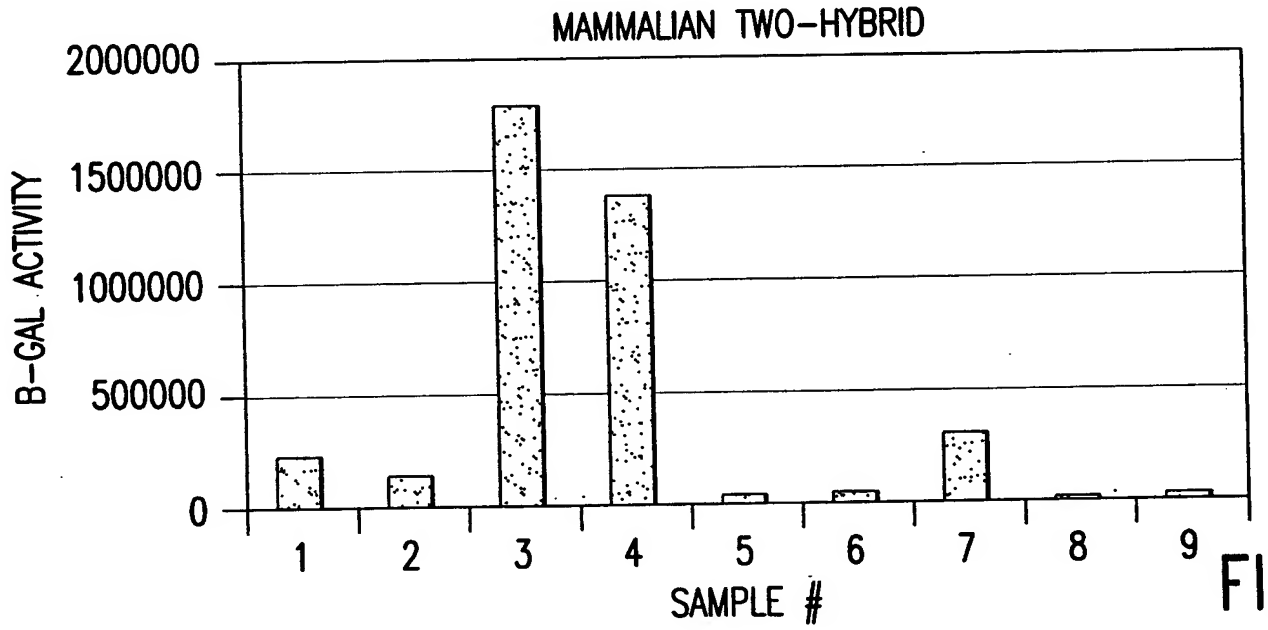

FIG. 10B

Inventor(s): Chesnut *et al.*; Tel: 202/371-2600
Title: Method and Compositions for Synthesis of Nucleic Acid...

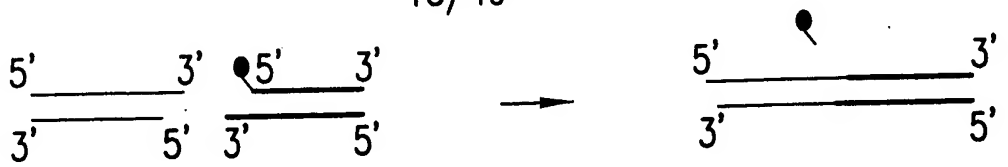

FIG. 11A

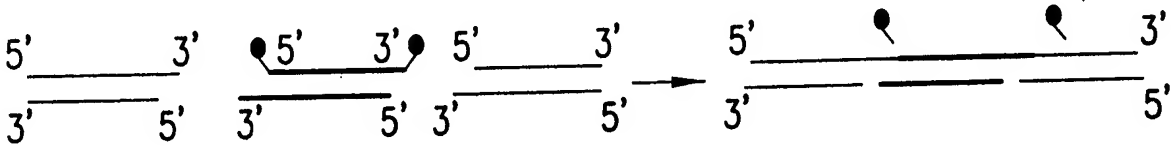

FIG. 11B

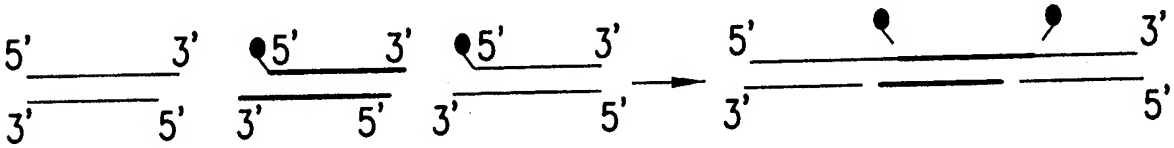

FIG. 11C

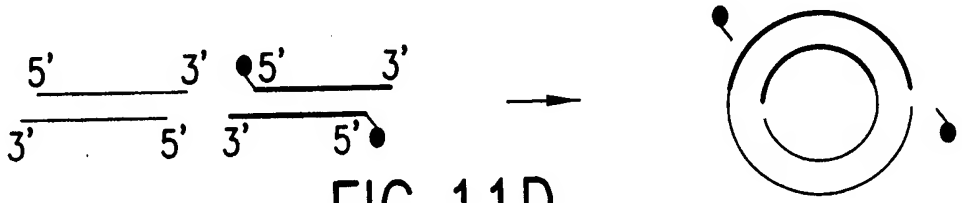

FIG. 11D

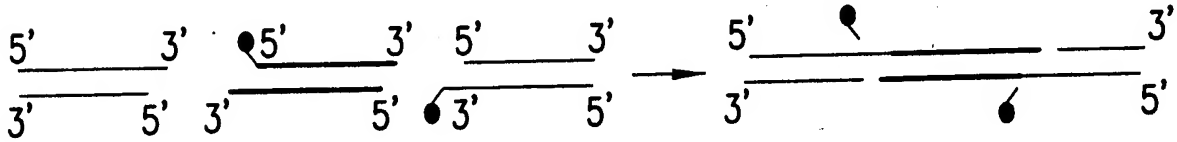

FIG. 11E

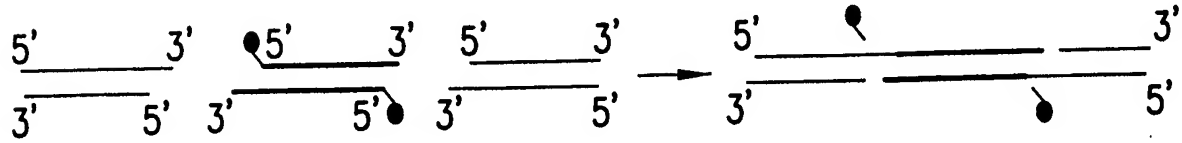

FIG. 11F

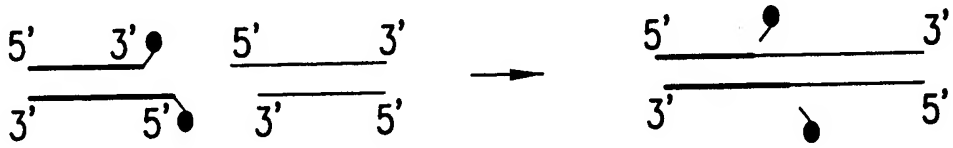

FIG. 12A

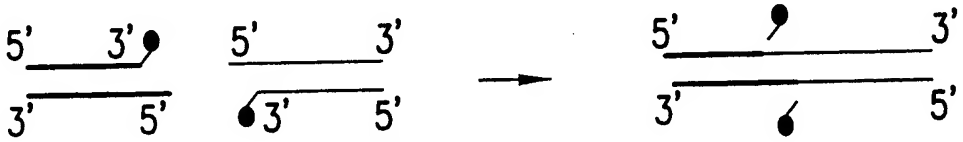

FIG. 12B

FIG. 12C

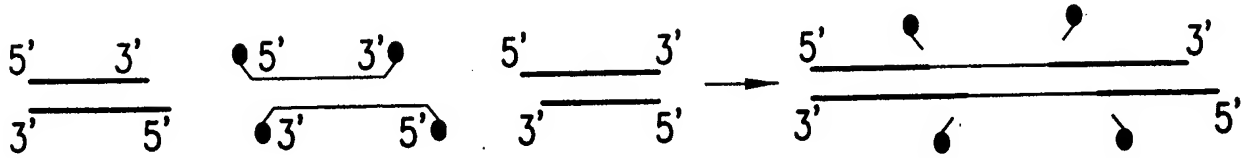

FIG. 12D

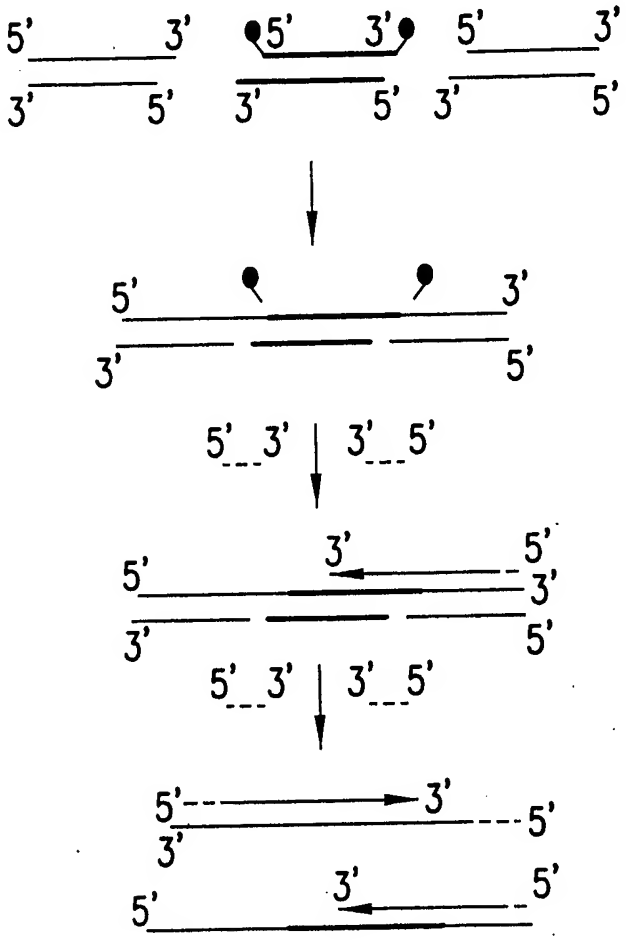

FIG. 13

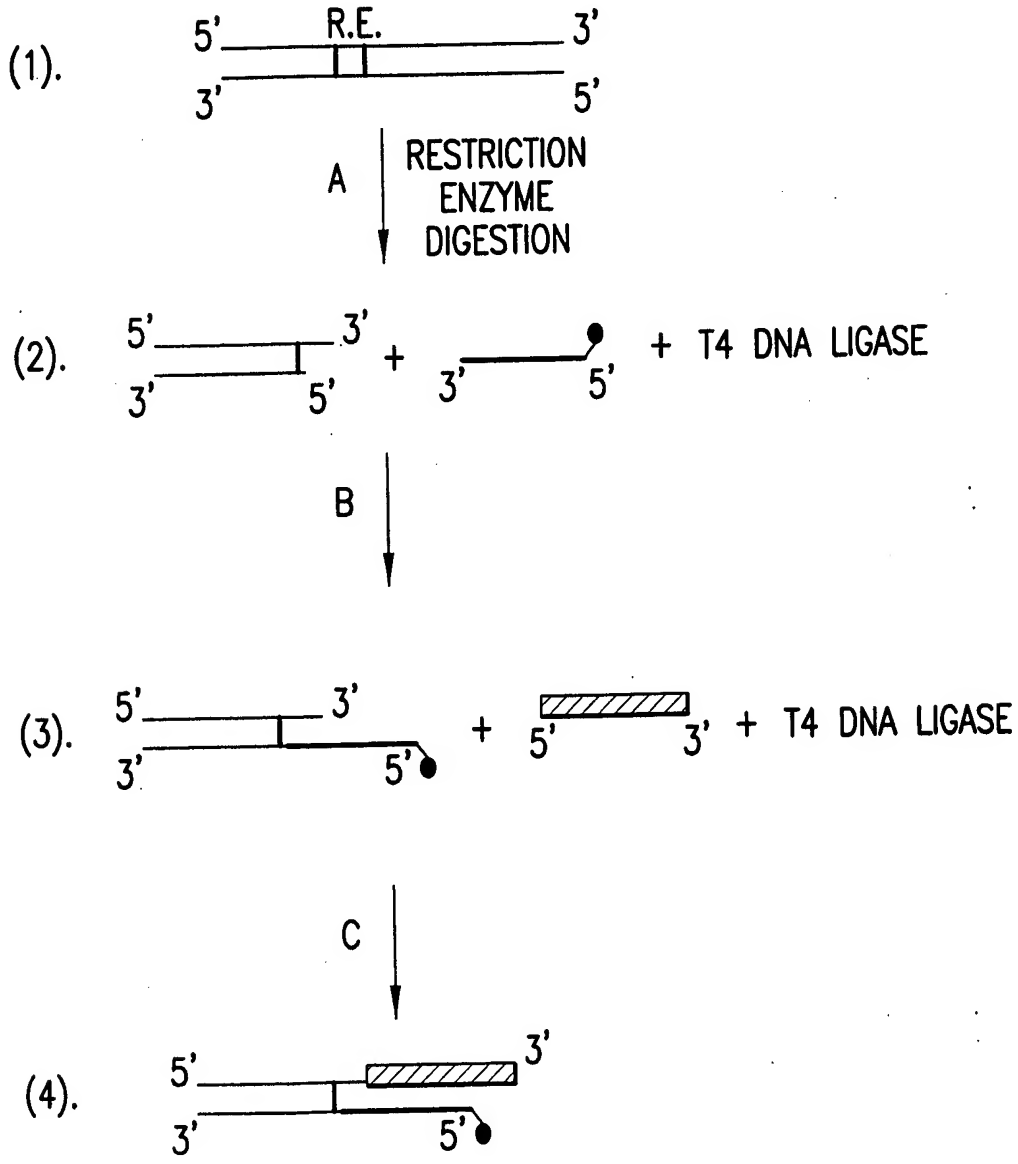
(1).
5'
R.E.
3'
3'
5'
A
RESTRICTION ENZYME DIGESTION
(2).
5'
3'
3'
5'
+
3'
5'
+ T4 DNA LIGASE
B
(3).
5'
3'
3'
5'
+
5'
3'
+ T4 DNA LIGASE
C
(4).
5'
3'
3'
5'

FIG. 14

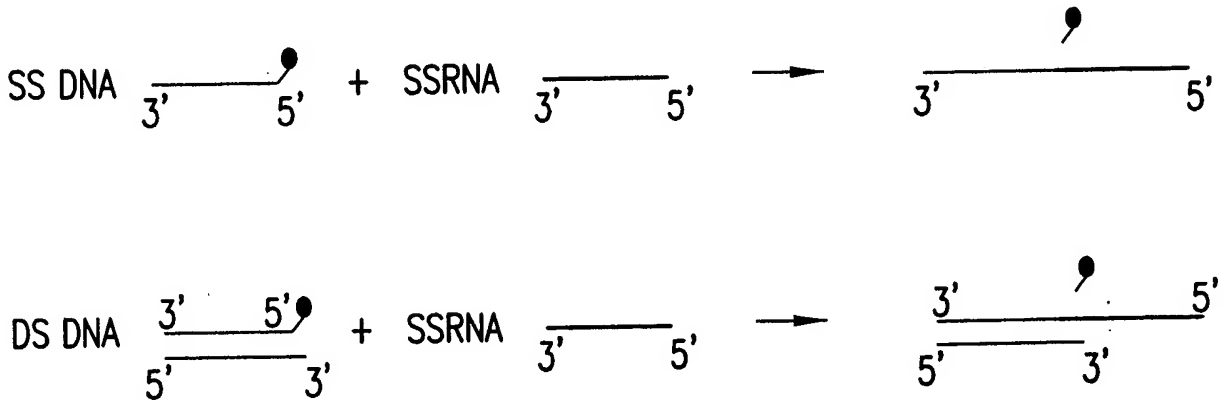

FIG. 15

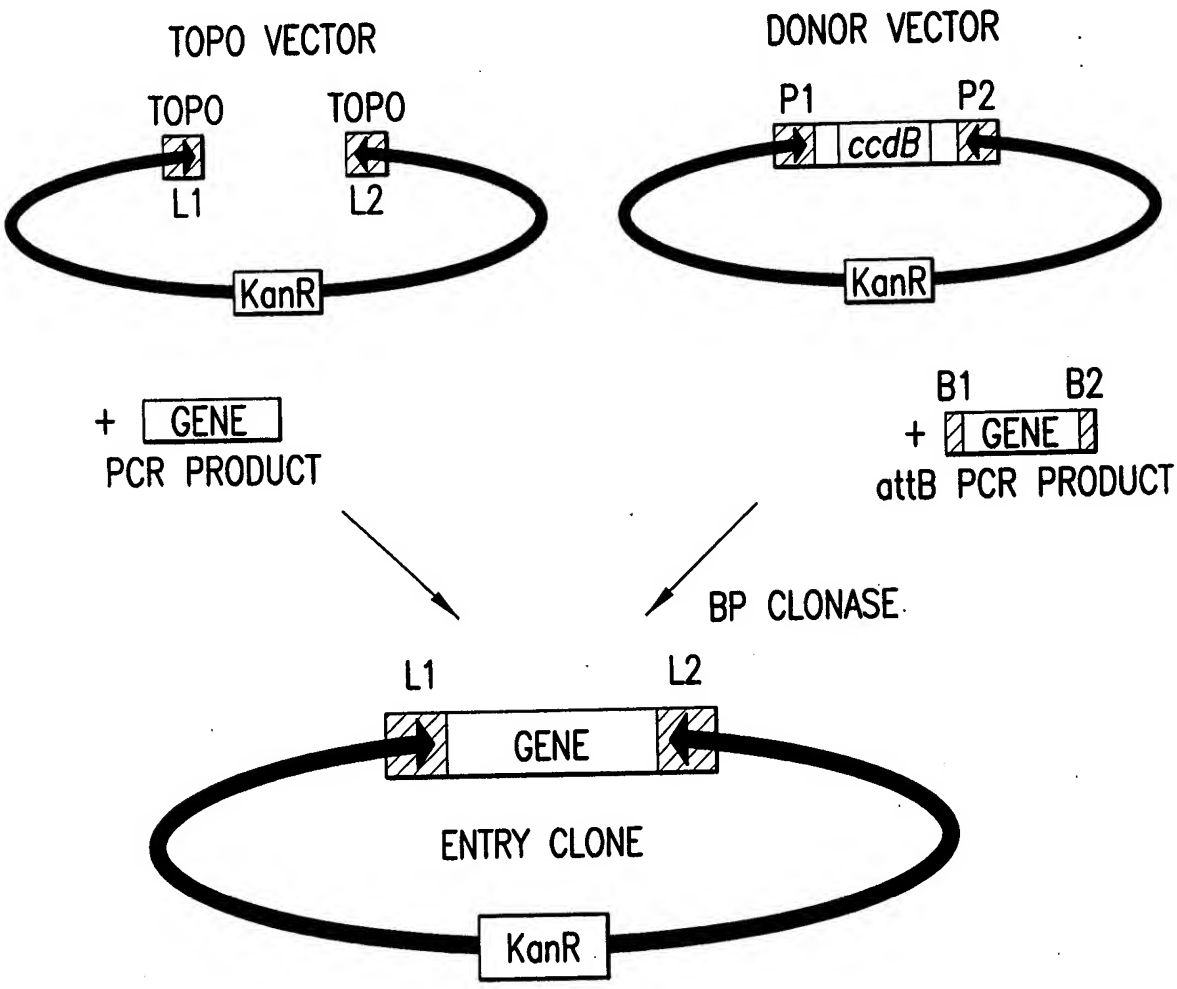
TOPO VECTOR
TOPO
TOPO
L1
L2
KanR
DONOR VECTOR
P1
P2
ccdB
KanR
+ GENE
PCR PRODUCT
B1
B2
+ GENE
attB PCR PRODUCT
BP CLONASE
L1
L2
GENE
ENTRY CLONE
KanR

FIG. 16

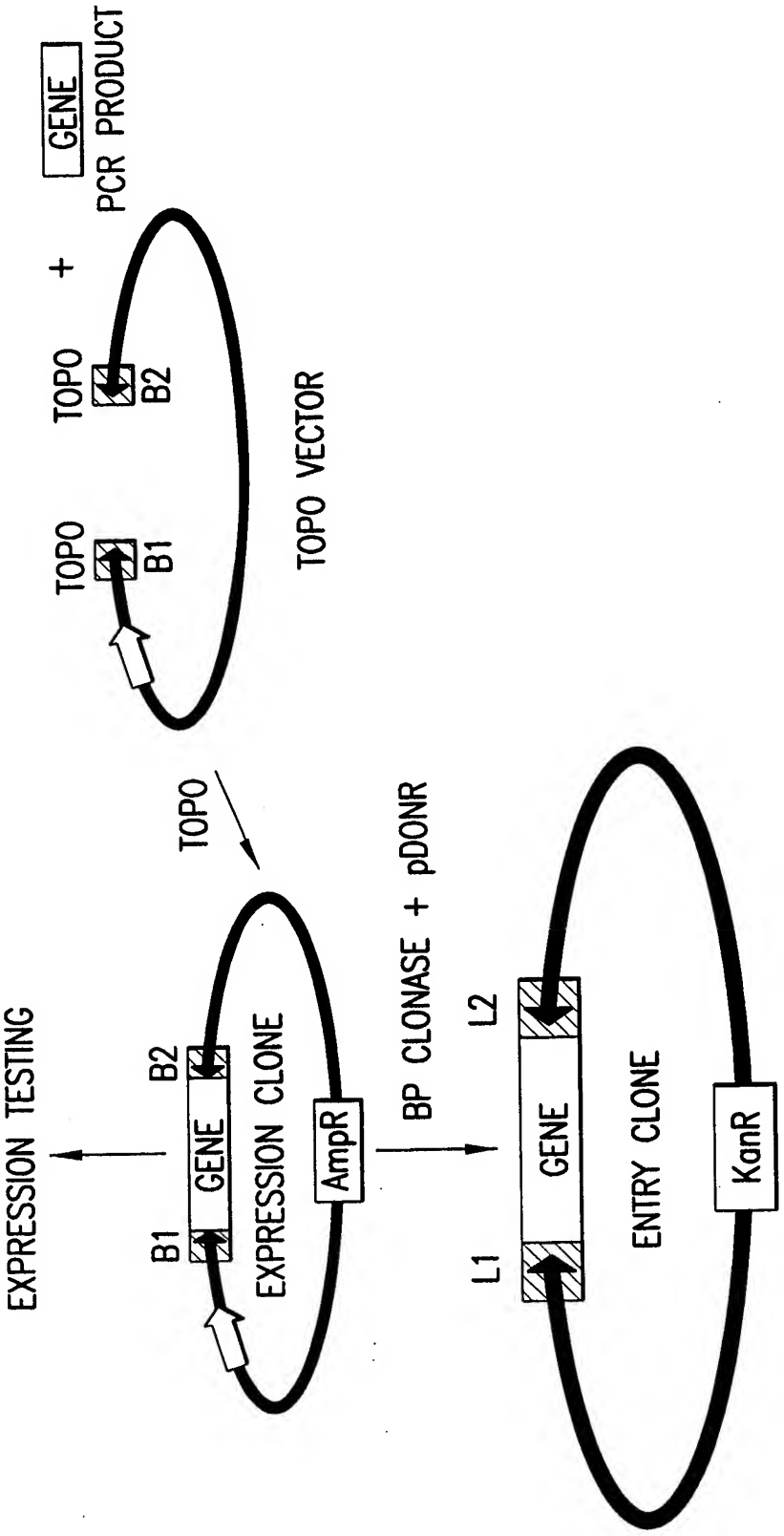
GENE
PCR PRODUCT
+
TOPO
B2
TOPO
B1
TOPO VECTOR
TOPO
BP CLONASE + pDONR
L2
L1
GENE
ENTRY CLONE
KanR
B2
B1
GENE
EXPRESSION CLONE
AmpR
EXPRESSION TESTING

FIG. 17

Inventor(s): Chesnut *et al.*; Tel: 202/371-2600
Title: Method and Compositions for Synthesis of Nucleic Acid...

MCS FOR pcDNAGW-DT(sc) AND pENTR-DT(sc)

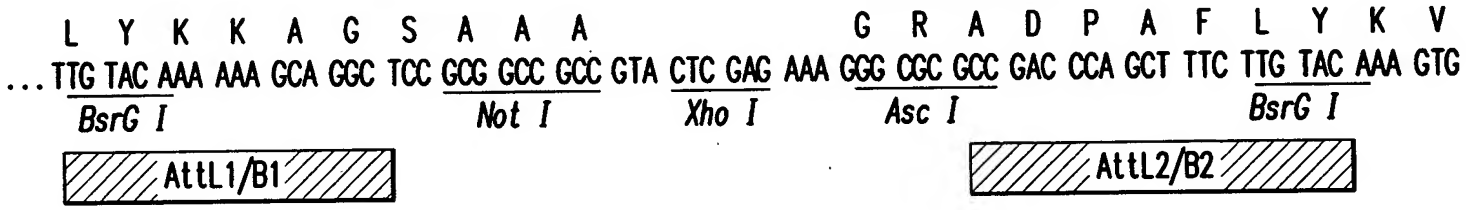

FIG. 18

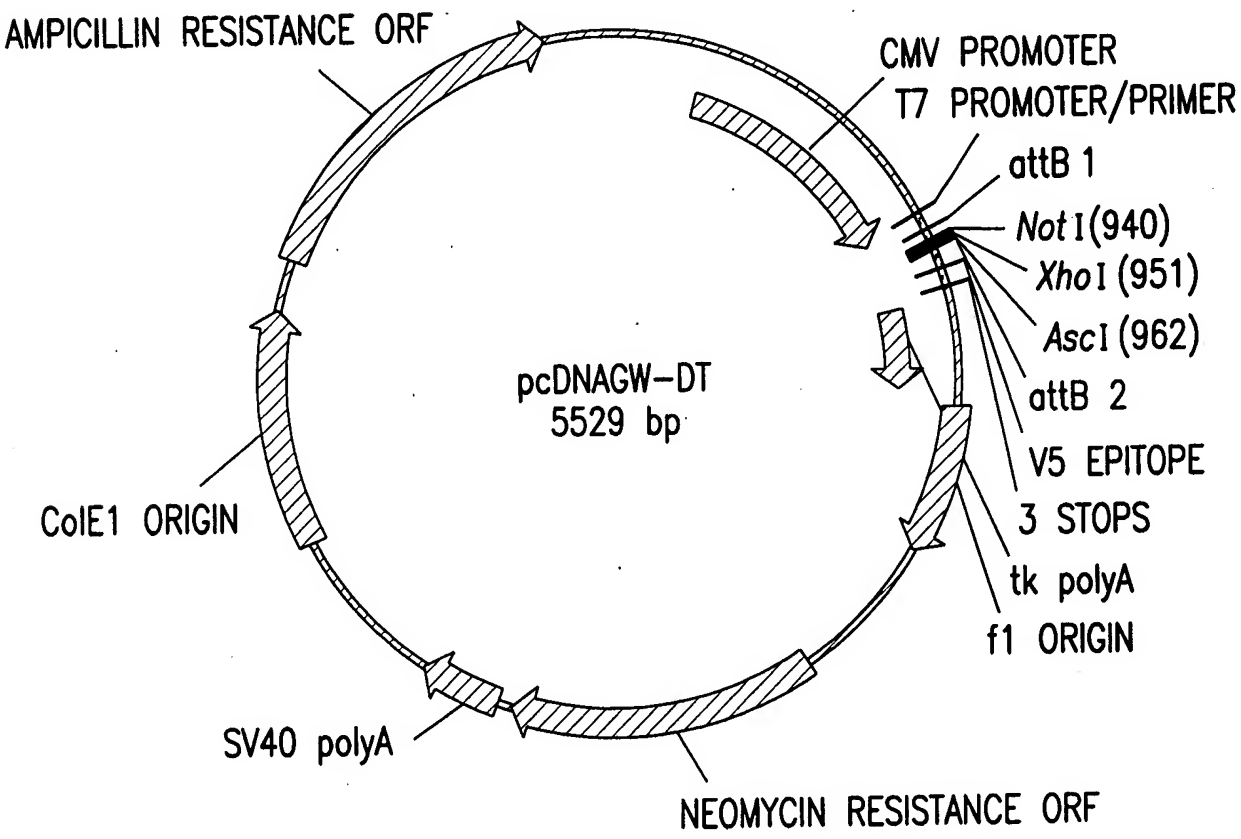
AMPICILLIN RESISTANCE ORF
CMV PROMOTER
T7 PROMOTER/PRIMER
attB 1
NotI (940)
XhoI (951)
AscI (962)
attB 2
V5 EPITOPE
3 STOPS
tk polyA
f1 ORIGIN
pcDNAGW-DT
5529 bp
ColE1 ORIGIN
SV40 polyA
NEOMYCIN RESISTANCE ORF

FIG. 19

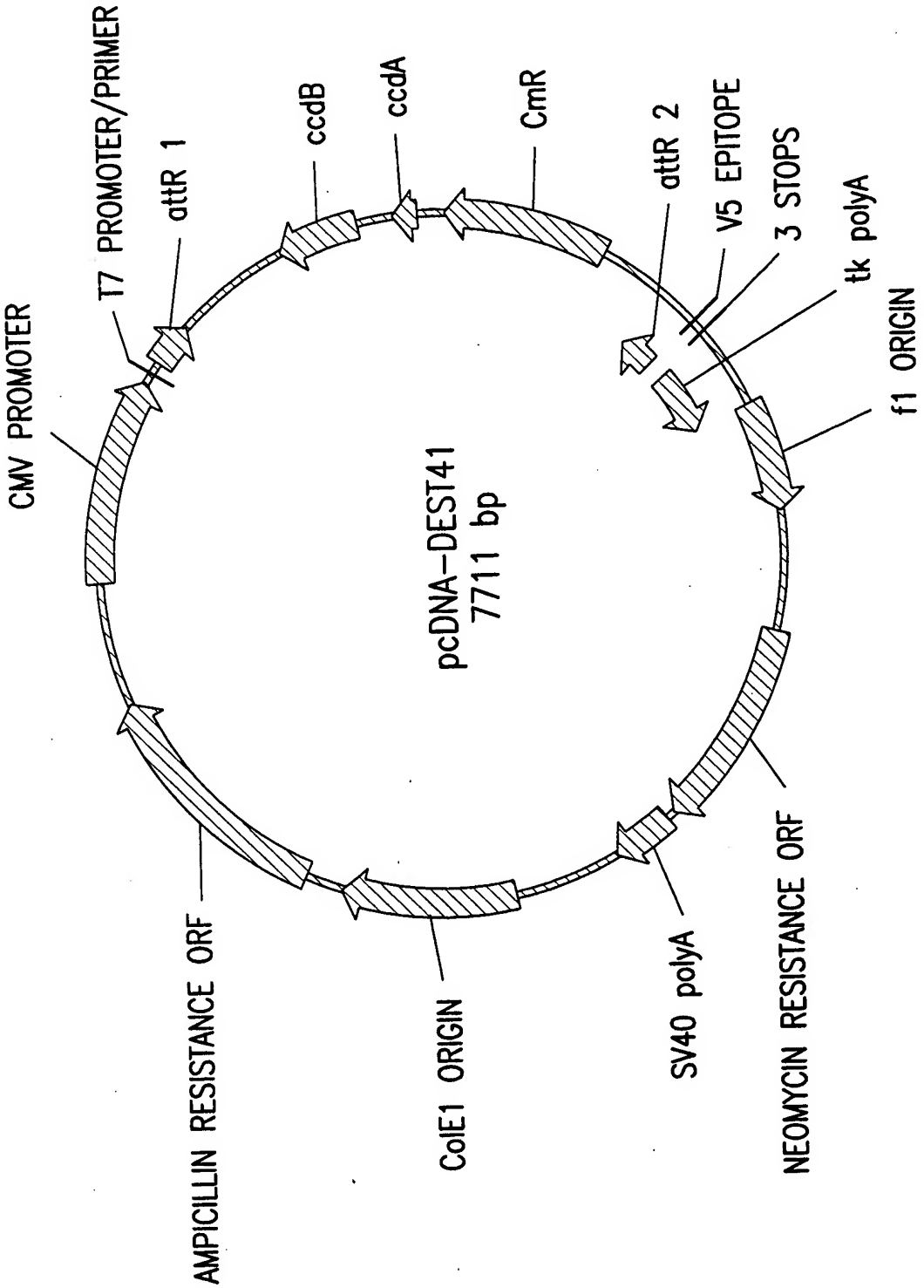
CMV PROMOTER
T7 PROMOTER/PRIMER
attR 1
ccdB
ccdA
CmR
attR 2
V5 EPITOPE
3 STOPS
tk polyA
f1 ORIGIN
pcDNA-DEST41
7711 bp
AMPICILLIN RESISTANCE ORF
ColE1 ORIGIN
SV40 polyA
NEOMYCIN RESISTANCE ORF

FIG. 20

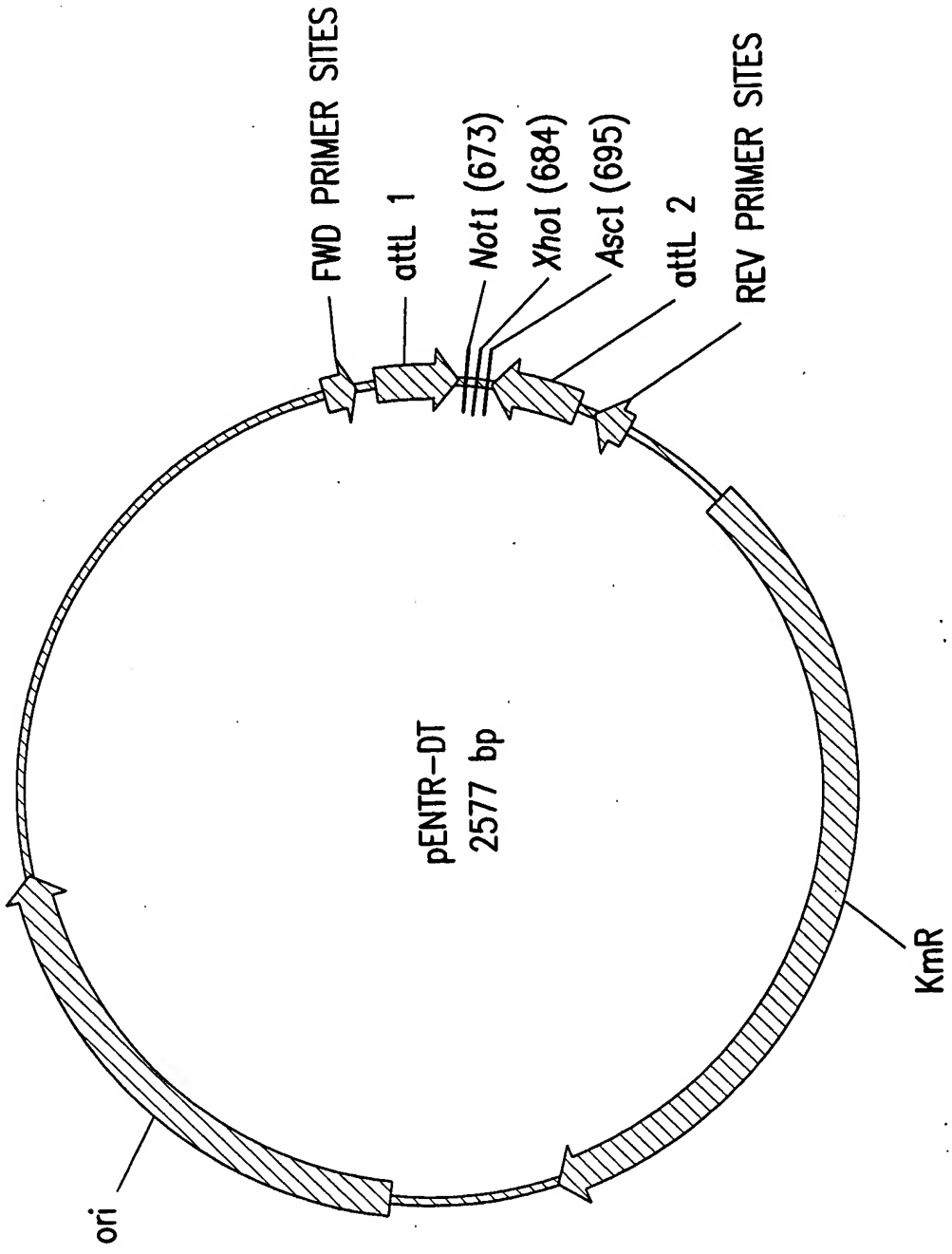
FWD PRIMER SITES
attL 1
NotI (673)
XhoI (684)
AscI (695)
attL 2
REV PRIMER SITES
pENTR-DT
2577 bp
KmR
ori

FIG. 21

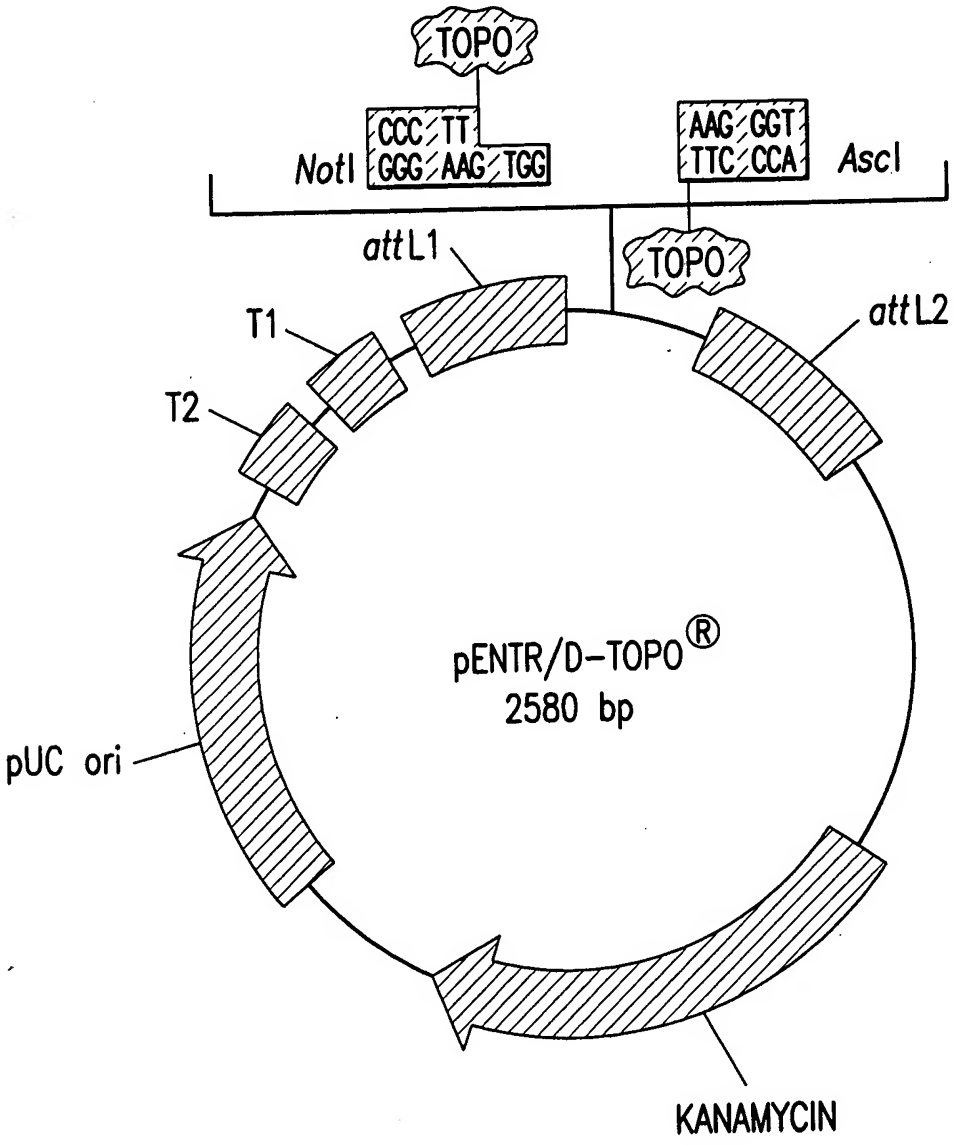
TOPO
CCC TT
GGG AAG TGG
NotI
AAG GGT
TTC CCA
AscI
attL1
TOPO
attL2
T1
T2
pENTR/D-TOPO®
2580 bp
pUC ori
KANAMYCIN

FIG. 22A

```
   1 ctttcctgcg ttatcccctg attctgtgga taaccgtatt accgcctttg agtgagctga
  61 taccgctcgc cgcagccgaa cgaccgagcg cagcgagtca gtgagcgagg aagcggaaga
 121 gcgcccaata cgcaaaccgc ctctccccgc gcgttggccg attcattaat gcagctggca
 181 cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc
 241 tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta
 301 gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc
 361 acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa
 421 caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg
 481 gcagttccct actctcgcgt taacgctagc atggatgttt tcccagtcac gacgttgtaa
 541 aacgacggcc agtcttaagc tcgggcccca aataatgatt ttattttgac tgatagtgac
 601 ctgttcgttg caacaaattg atgagcaatg ctttttttata atgccaactt tgtacaaaaa
 661 agcaggctcc gcggccgccc cttcaccatg nnnnnnnnna agggtgggcg cgccgaccca
 721 gctttcttgt acaaagttgg cattataaga aagcattgct tatcaatttg ttgcaacgaa
 781 caggtcacta tcagtcaaaa taaaatcatt atttgccatc cagctgatat cccctatagt
 841 gagtcgtatt acatggtcat agctgtttcc tggcagctct ggcccgtgtc tcaaaatctc
 901 tgatgttaca ttgcacaaga taaaaatata tcatcatgaa caataaaact gtctgcttac
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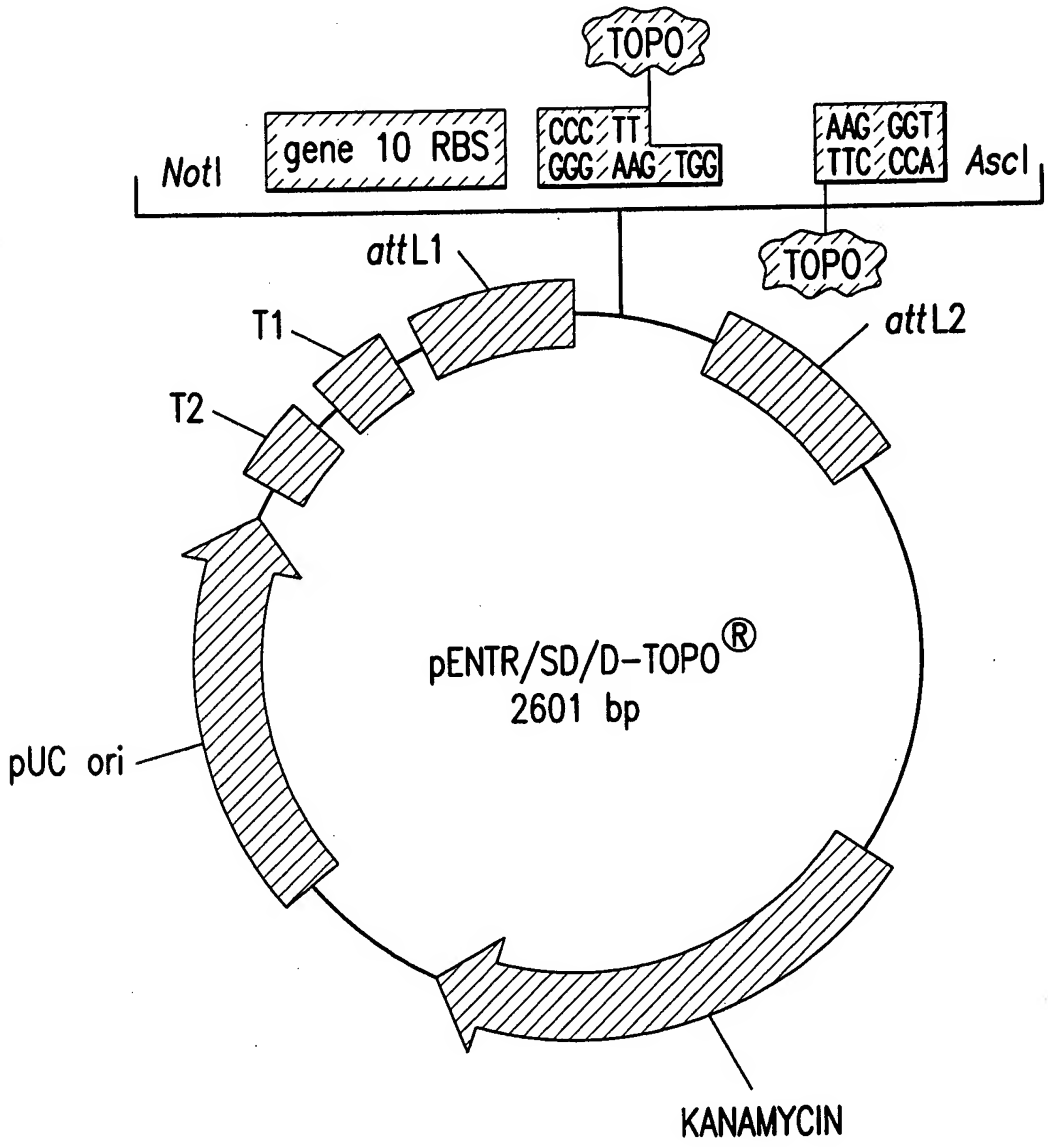
FIG.22B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AAG GGT
TTC CCA
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AscI
attL1
TOPO
T1
attL2
T2
pENTR/SD/D-TOPO®
2601 bp
pUC ori
KANAMYCIN

FIG. 23A

```
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 181 cgacaggttt cccgactgga aagcgggcag tgagcgcaac gcaattaata cgcgtaccgc
 241 tagccaggaa gagtttgtag aaacgcaaaa aggccatccg tcaggatggc cttctgctta
 301 gtttgatgcc tggcagttta tggcgggcgt cctgcccgcc accctccggg ccgttgcttc
 361 acaacgttca aatccgctcc cggcggattt gtcctactca ggagagcgtt caccgacaaa
 421 caacagataa aacgaaaggc ccagtcttcc gactgagcct ttcgttttat ttgatgcctg
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 781 aatttgttgc aacgaacagg tcactatcag tcaaaataaa atcattattt gccatccagc
 841 tgatatcccc tatagtgagt cgtattacat ggtcatagct gtttcctggc agctctggcc
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2521 gtcaggggggg cggagcctat ggaaaaacgc cagcaacgcg gccttttttac ggttcctggc
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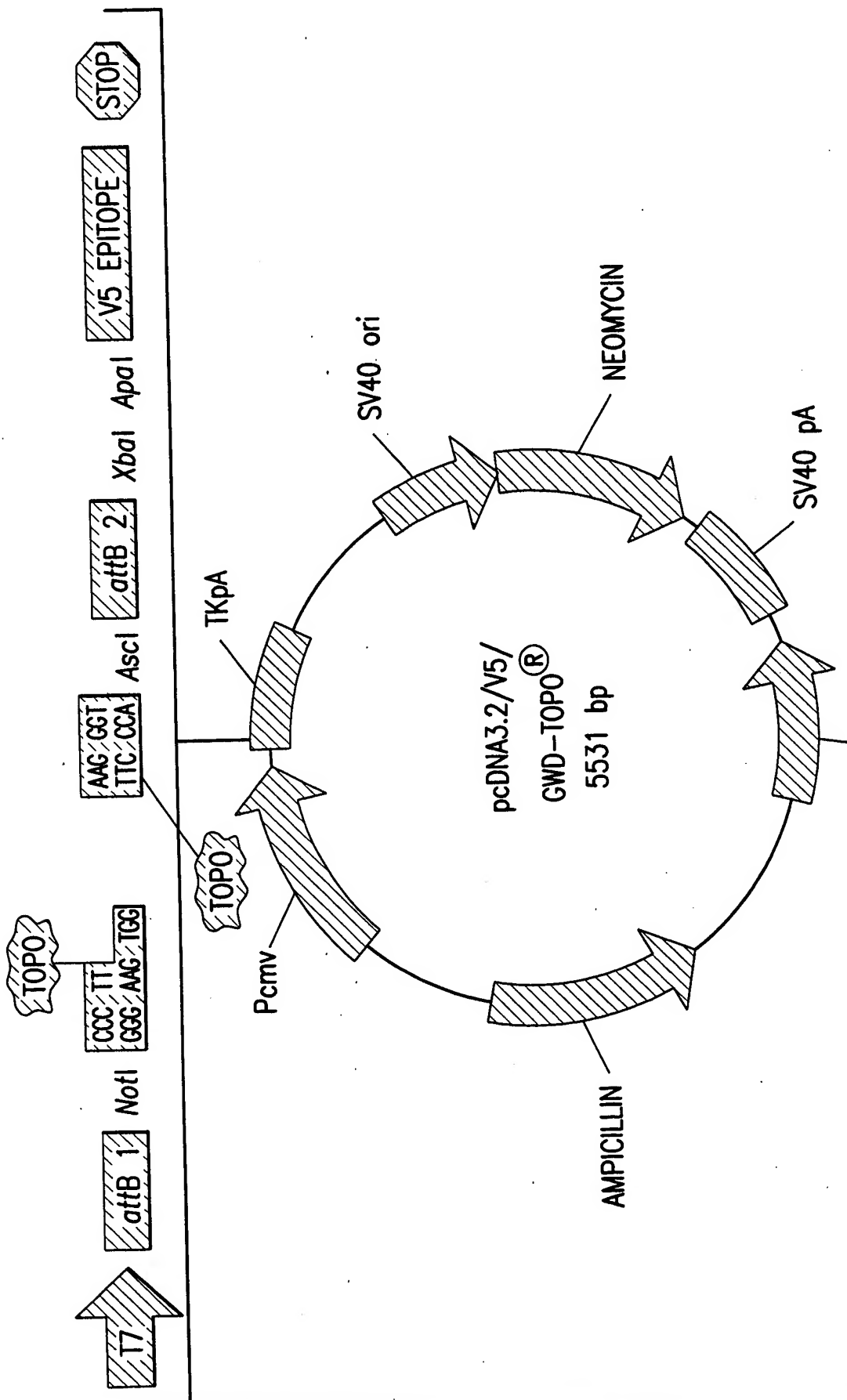
```

FIG.23B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NotI
CCC TT
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TOPO
TOPO
AAG GGT
TTC CCA
AscI
attB 2
XbaI
ApaI
V5 EPITOPE
STOP
TKpA
Pcmv
SV40 ori
NEOMYCIN
SV40 pA
pUC ori
AMPICILLIN
pcDNA3.2/V5/
GWD-TOPO®
5531 bp

FIG. 24A

```
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  61 ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg
 121 cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc
 181 ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt
 241 gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata
 301 tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc
 361 cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc
 421 attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt
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 601 tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg
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```

FIG.24B

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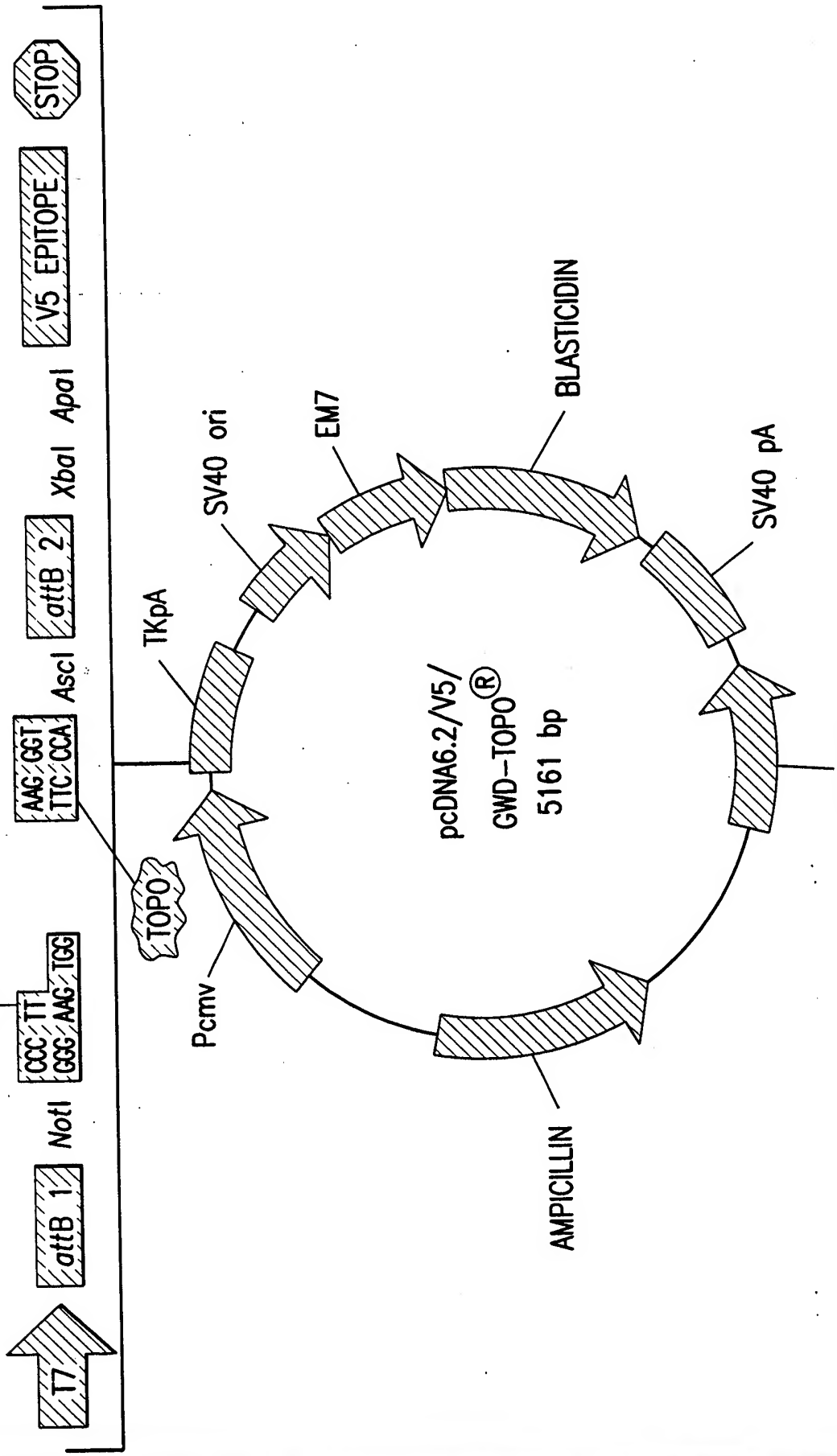
FIG.24C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V5 EPITOPE
STOP
TKpA
SV40 ori
EM7
BLASTICIDIN
SV40 pA
pUC ori
Pcmv
pcDNA6.2/V5/
GWD-TOPO®
5161 bp
AMPICILLIN

FIG. 25A

```
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2041 aactccgccc agttccgccc attctccgcc ccatggctga ctaatttttt ttatttatgc
2101 agaggccgag gccgcctctg cctctgagct attccagaag tagtgaggag gctttttttgg
2161 aggcctaggc ttttgcaaaa agctcccggg agcttgtata tccattttcg gatctgatca
2221 gcacgtgttg acaattaatc atcggcatag tatatcggca tagtataata cgacaaggtg
2281 aggaactaaa ccatggccaa gcctttgtct caagaagaat ccaccctcat tgaaagagca
2341 acggctacaa tcaacagcat ccccatctct gaagactaca gcgtcgccag cgcagctctc
2401 tctagcgacg gccgcatctt cactggtgtc aatgtatatc attttactgg gggaccttgt
2461 gcagaactcg tggtgctggg cactgctgct gctgcggcag ctggcaacct gacttgtatc
2521 gtcgcgatcg gaaatgagaa caggggcatc ttgagccccct gcggacggtg ccgacaggtg
2581 cttctcgatc tgcatcctgg gatcaaagcc atagtgaagg acagtgatgg acagccgacg
2641 gcagttggga ttcgtgaatt gctgccctct ggttatgtgt gggagggcta agcacttcgt
2701 ggccgaggag caggactgac acgtgctacg agatttcgat tccaccgccg ccttctatga
2761 aaggttgggc ttcggaatcg ttttccggga cgccggctgg atgatcctcc agcgcgggga-
```

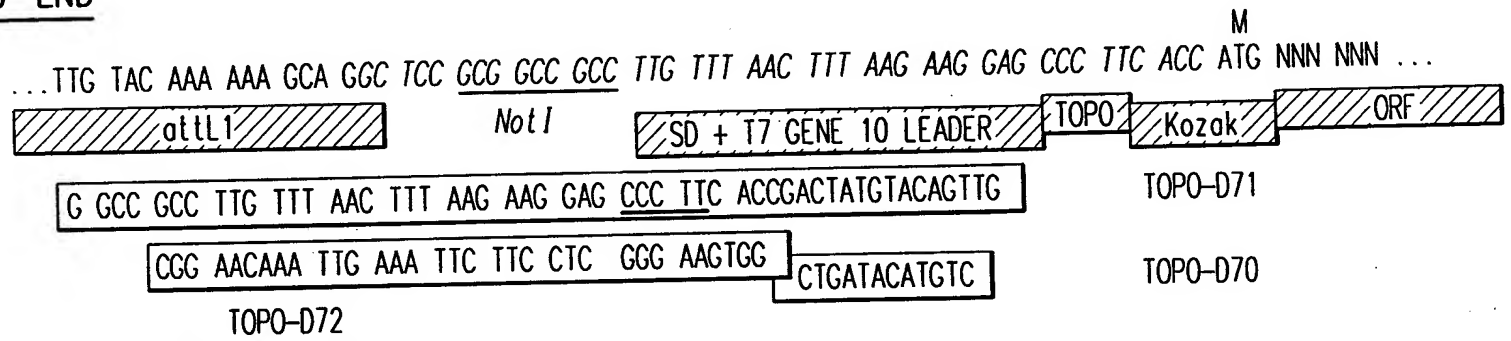
FIG.25B

2821 tctcatgctg gagttcttcg cccaccccaa cttgtttatt gcagcttata atggttacaa
2881 ataaagcaat agcatcacaa atttcacaaa taaagcattt ttttcactgc attctagttg
2941 tggtttgtcc aaactcatca atgtatctta tcatgtctgt ataccgtcga cctctagcta
3001 gagcttggcg taatcatggt catagctgtt tcctgtgtga aattgttatc cgctcacaat
3061 tccacacaac atacgagccg gaagcataaa gtgtaaagcc tggggtgcct aatgagtgag
3121 ctaactcaca ttaattgcgt tgcgctcact gcccgctttc cagtcgggaa acctgtcgtg
3181 ccagctgcat taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta ttgggcgctc
3241 ttccgcttcc tcgctcactg actcgctgcg ctcggtcgtt cggctgcggc gagcggtatc
3301 agctcactca aaggcggtaa tacggttatc cacagaatca ggggataacg caggaaagaa
3361 catgtgagca aaaggccagc aaaaggccag gaaccgtaaa aaggccgcgt tgctggcgtt
3421 tttccatagg ctccgccccc ctgacgagca tcacaaaaat cgacgctcaa gtcagaggtg
3481 gcgaaacccg acaggactat aaagatacca ggcgtttccc cctggaagct ccctcgtgcg
3541 ctctcctgtt ccgaccctgc cgcttaccgg atacctgtcc gcctttctcc cttcgggaag
3601 cgtggcgctt tctcatagct cacgctgtag gtatctcagt tcggtgtagg tcgttcgctc
3661 caagctgggc tgtgtgcacg aacccccccgt tcagcccgac cgctgcgcct tatccggtaa
3721 ctatcgtctt gagtccaacc cggtaagaca cgacttatcg ccactggcag cagccactgg
3781 taacaggatt agcagagcga ggtatgtagg cggtgctaca gagttcttga agtggtggcc
3841 taactacggc tacactagaa gaacagtatt tggtatctgc gctctgctga agccagttac
3901 cttcggaaaa agagttggta gctcttgatc cggcaaacaa accaccgctg gtagcggttt
3961 ttttgtttgc aagcagcaga ttacgcgcag aaaaaaagga tctcaagaag atcctttgat
4021 cttttctacg gggtctgacg ctcagtggaa cgaaaactca cgttaaggga ttttggtcat
4081 gagattatca aaaaggatct tcacctagat ccttttaaat taaaaatgaa gttttaaatc
4141 aatctaaagt atatatgagt aaacttggtc tgacagttac caatgcttaa tcagtgaggc
4201 acctatctca gcgatctgtc tatttcgttc atccatagtt gcctgactcc ccgtcgtgta
4261 gataactacg atacgggagg gcttaccatc tggccccagt gctgcaatga taccgcgaga
4321 cccacgctca ccggctccag atttatcagc aataaaccag ccagccggaa gggccgagcg
4381 cagaagtggt cctgcaactt tatccgcctc catccagtct attaattgtt gccgggaagc
4441 tagagtaagt agttcgccag ttaatagttt gcgcaacgtt gttgccattg ctacaggcat
4501 cgtggtgtca cgctcgtcgt ttggtatggc ttcattcagc tccggttccc aacgatcaag
4561 gcgagttaca tgatccccca tgttgtgcaa aaaagcggtt agctccttcg gtcctccgat
4621 cgttgtcaga agtaagttgg ccgcagtgtt atcactcatg gttatggcag cactgcataa
4681 ttctcttact gtcatgccat ccgtaagatg cttttctgtg actggtgagt actcaaccaa
4741 gtcattctga gaatagtgta tgcggcgacc gagttgctct tgcccggcgt caatacggga
4801 taataccgcg ccacatagca gaactttaaa agtgctcatc attggaaaac gttcttcggg
4861 gcgaaaactc tcaaggatct taccgctgtt gagatccagt tcgatgtaac ccactcgtgc
4921 acccaactga tcttcagcat cttttacttt caccagcgtt tctgggtgag caaaaacagg
4981 aaggcaaaat gccgcaaaaa agggaataag ggcgacacgg aaatgttgaa tactcatact
5041 cttccttttt caatattatt gaagcattta tcagggttat tgtctcatga gcggatacat
5101 atttgaatgt atttagaaaa ataaacaaat aggggttccg cgcacatttc cccgaaaagt
5161 gccacctgac gtc

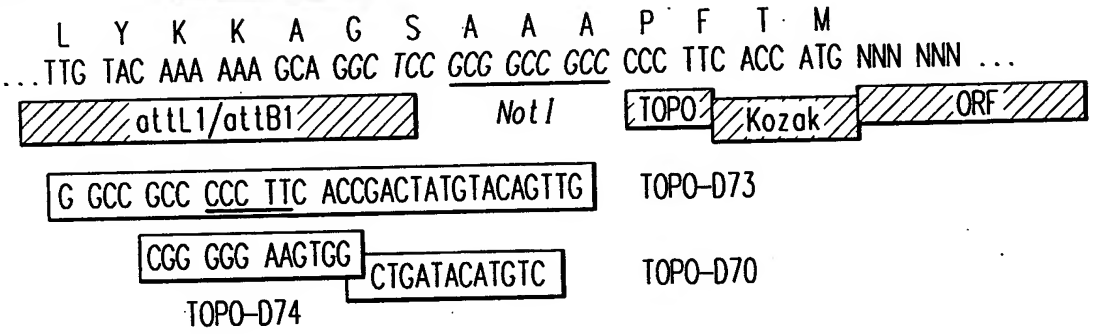
FIG.25C

Inventor(s): Chesnut *et al.*; Tel: 202/371-2600
Title: Method and Compositions for Synthesis of Nucleic Acid...

pENTR/SD-dTopo: 5' END



pENTR-dTOPO AND pcDNAGW-dTOPO: 5' END



pENTR/SD-dTOPO, pENTR-dTOPO, AND pcDNAGW-dTOPO: 3' END

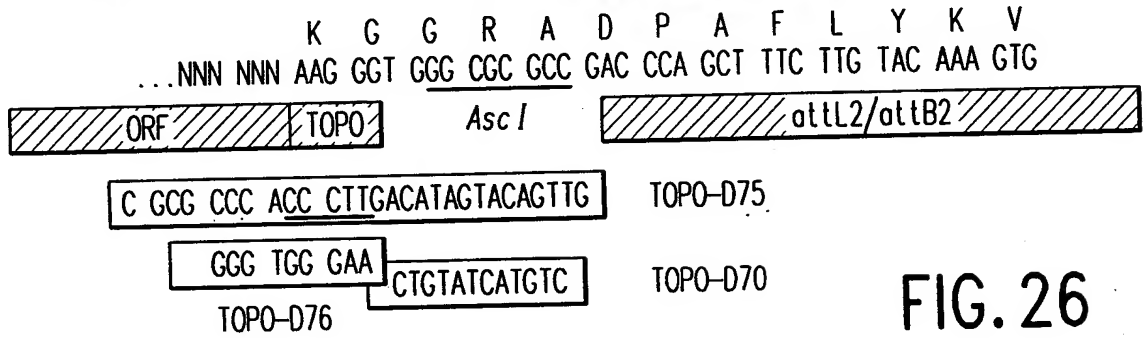


FIG. 26

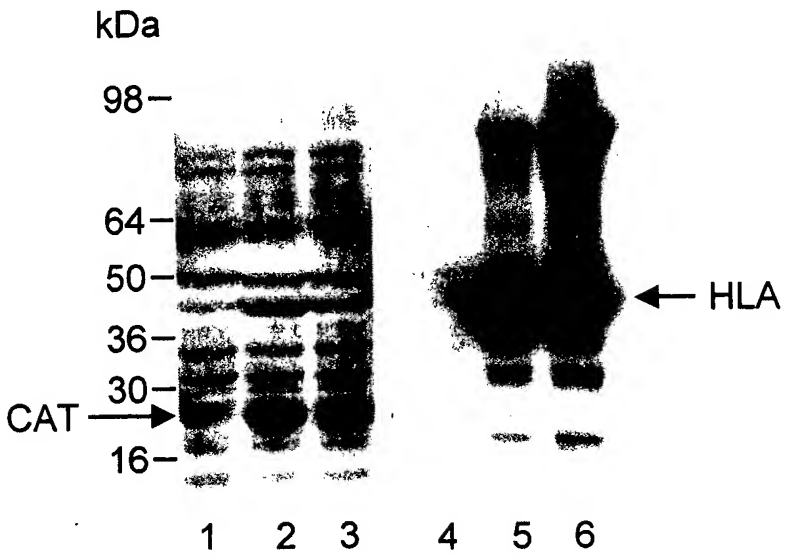


FIG.27

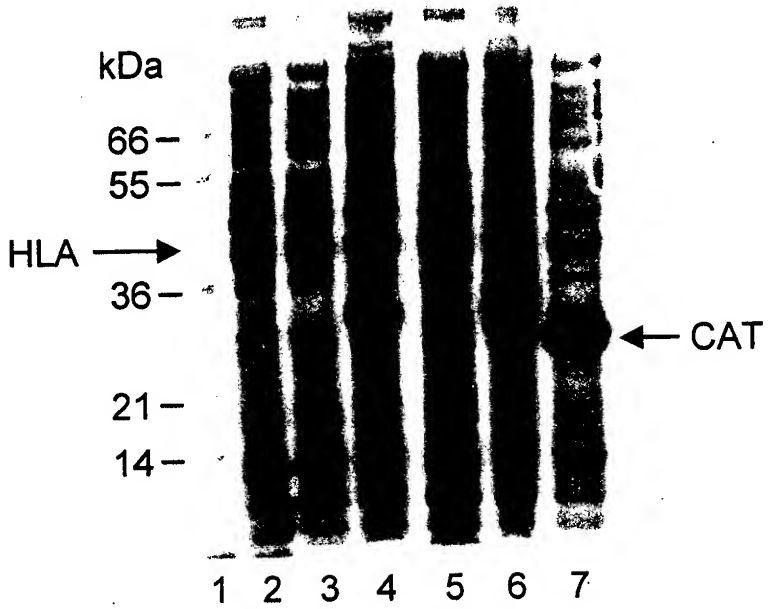


FIG.28

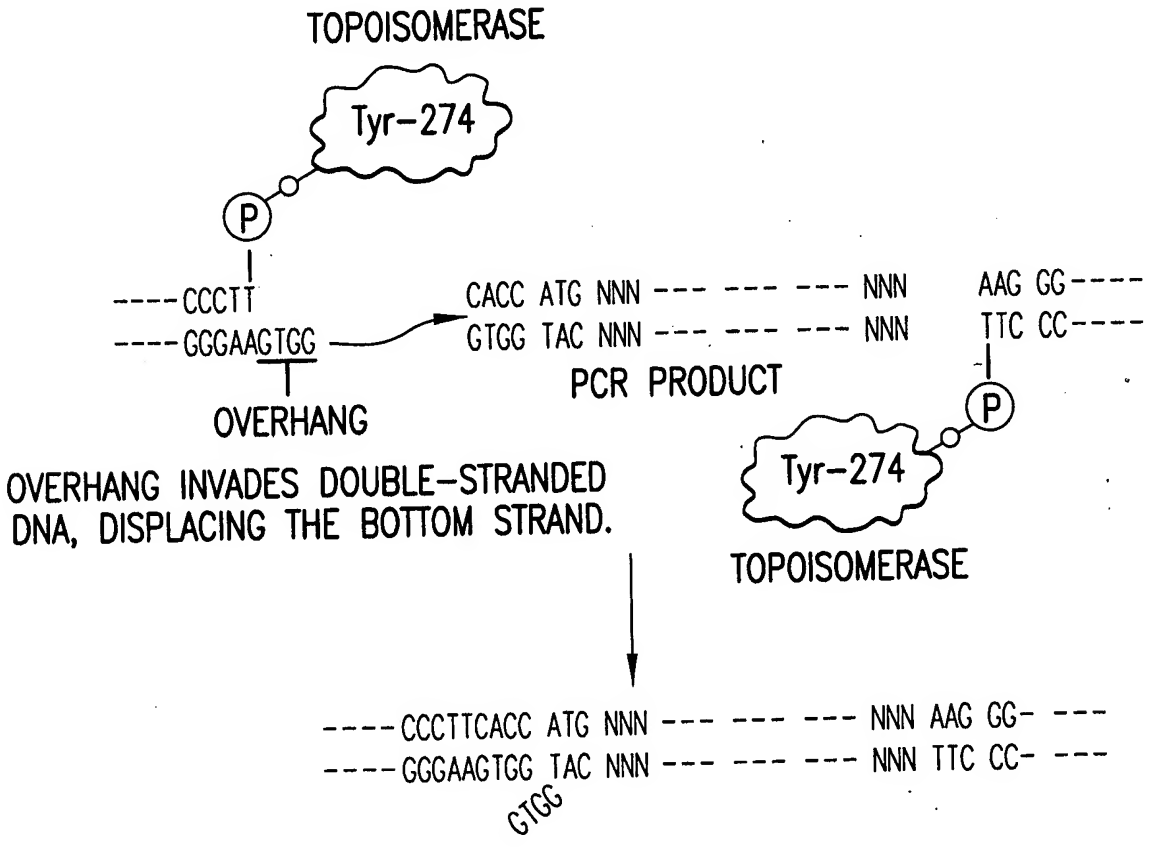
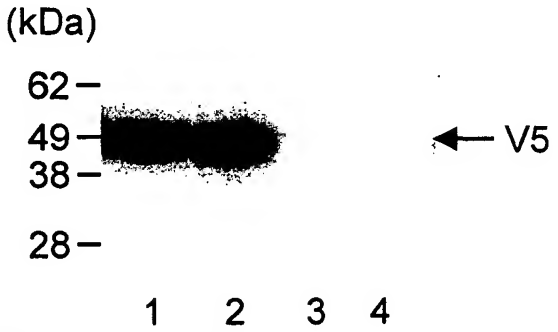
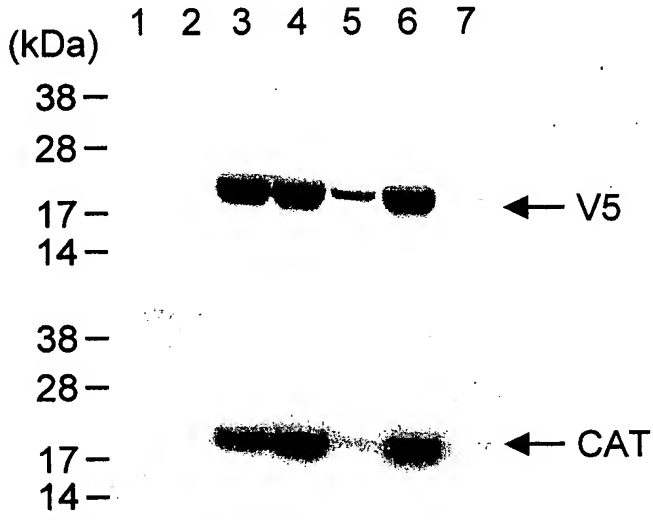

TOPOISOMERASE
Tyr-274
P
----CCCTT
----GGGAAGTGG
OVERHANG
CACC ATG NNN --- --- --- NNN
GTGG TAC NNN --- --- --- NNN
AAG GG----
TTC CC----
PCR PRODUCT
P
Tyr-274
TOPOISOMERASE
OVERHANG INVADES DOUBLE-STRANDED DNA, DISPLACING THE BOTTOM STRAND.
----CCCTTCACC ATG NNN --- --- --- NNN AAG GG- ---
----GGGAAGTGG TAC NNN --- --- --- NNN TTC CC- ---
GTGG

FIG. 29



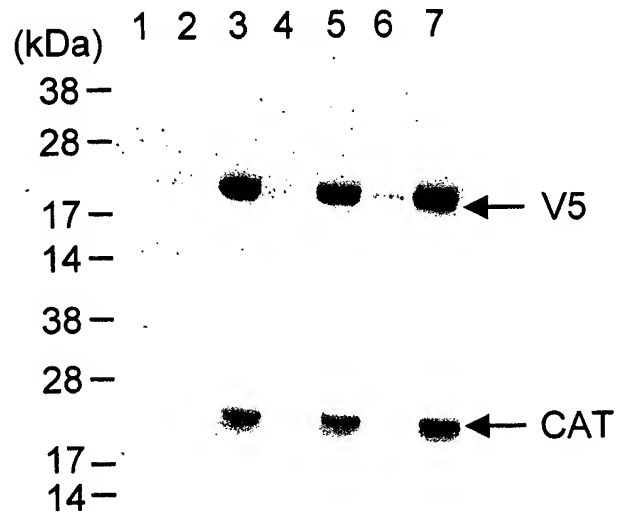
Lane 1: pCMVTetO/CAT/V5TKpA (without secondary PCR) + Tet
Lane 2: pCMVTetO/CAT/V5TKpA (with secondary PCR) + Tet
Lane 3: pCMVTetO/CAT/V5TKpA (with secondary PCR) - Tet
Lane 4: pCMVTetO/CAT/V5TKpA (without secondary PCR) - Tet

FIG.30A



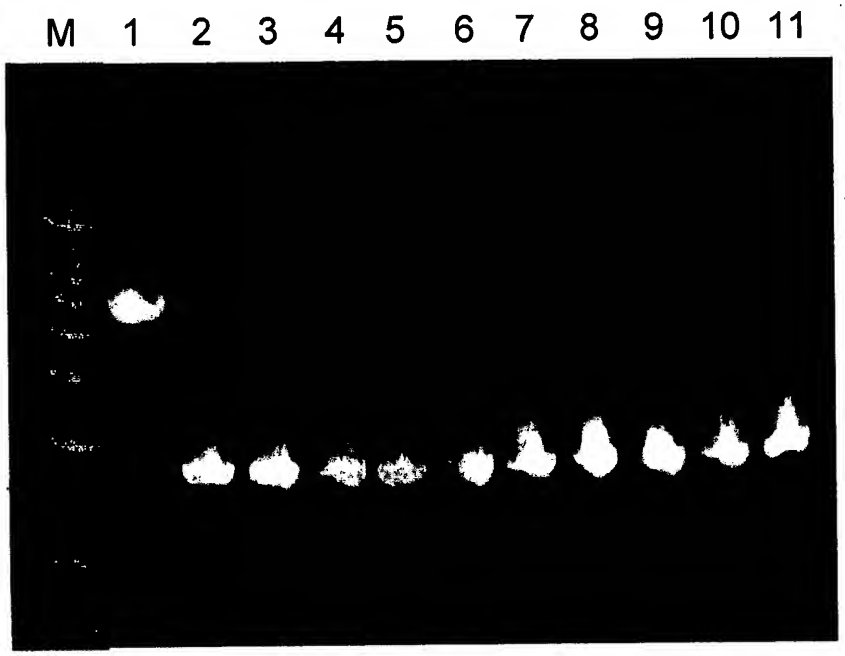
Lane 1: TRex-CHO Cells + Tet
Lane 2: without secondary PCR (with purified CAT) - Tet
Lane 3: without secondary PCR (with purified CAT) + Tet
Lane 4: without secondary PCR (with unpurified CAT) + Tet
Lane 5: without secondary PCR (with unpurified CAT) -Tet
Lane 6: with secondary PCR + Tet
Lane 7: with secondary PCR - Tet

FIG.30B



Lane 1: TRex-293 Cells + Tet
Lane 2: without secondary PCR (with purified CAT) - Tet
Lane 3: without secondary PCR (with purified CAT) + Tet
Lane 4: without secondary PCR (with unpurified CAT) - Tet
Lane 5: without secondary PCR (with unpurified CAT) +Tet
Lane 6: with secondary PCR - Tet
Lane 7: with secondary PCR + Tet

FIG.30C



Lane 1: negative control; lanes 2-11: test clones; M: 500 bp marker

FIG.31

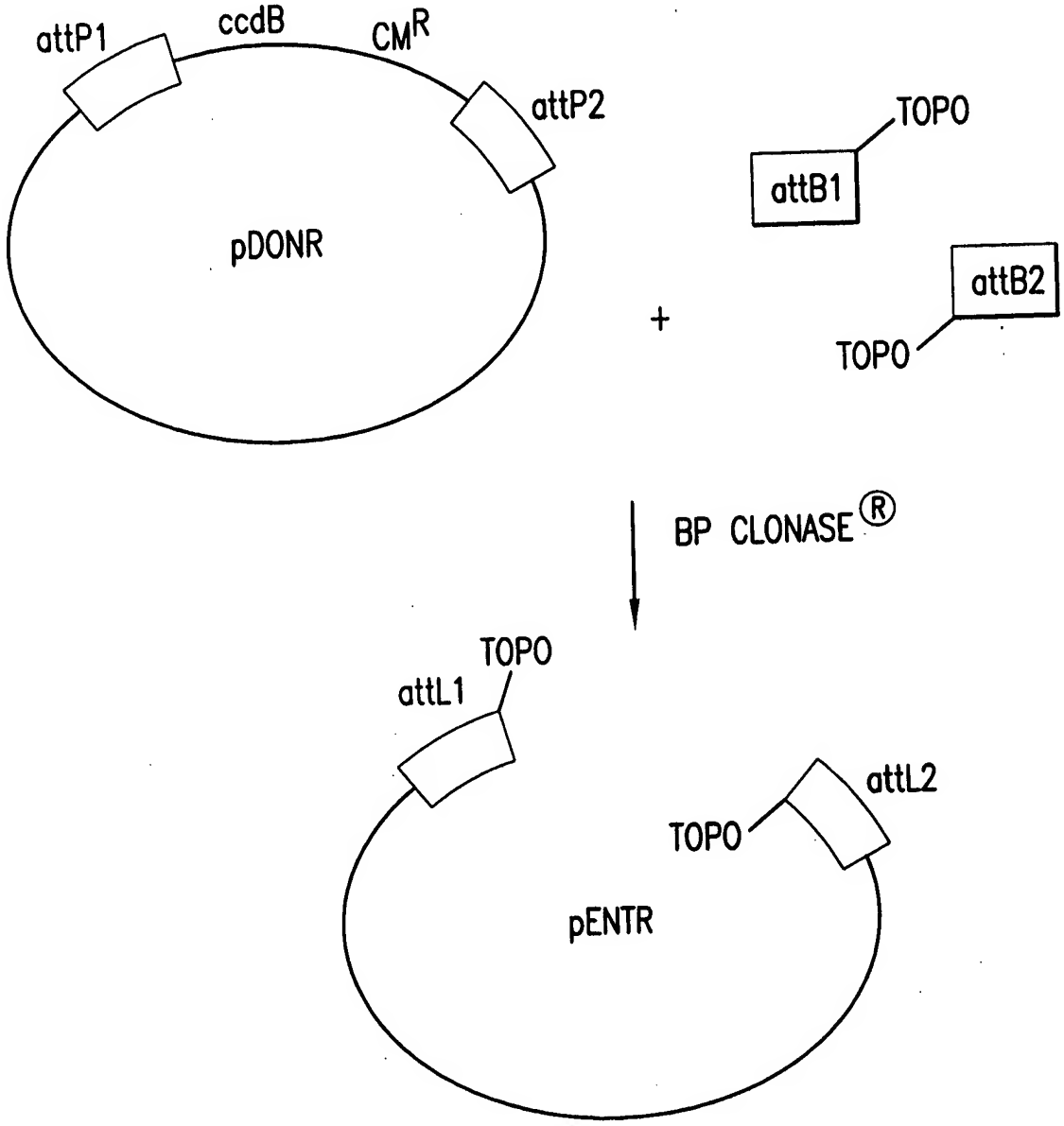

attP1
ccdB
CMR
attP2
pDONR
+
TOPO
attB1
attB2
TOPO
BP CLONASE®
TOPO
attL1
attL2
TOPO
pENTR

FIG. 32

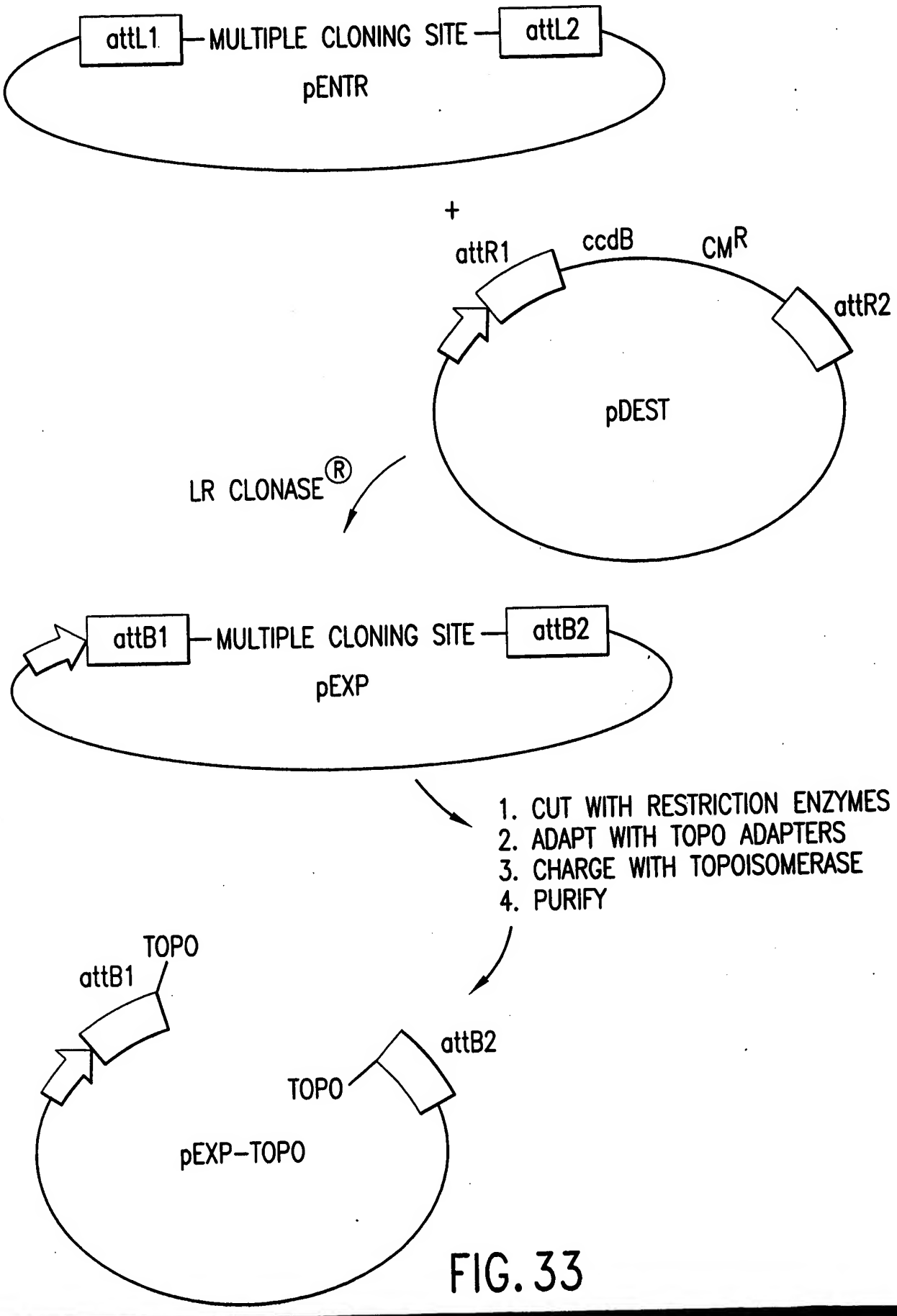

attL1
MULTIPLE CLONING SITE
attL2
pENTR
+
attR1
ccdB
CMR
attR2
pDEST
LR CLONASE®
attB1
MULTIPLE CLONING SITE
attB2
pEXP
1. CUT WITH RESTRICTION ENZYMES
2. ADAPT WITH TOPO ADAPTERS
3. CHARGE WITH TOPOISOMERASE
4. PURIFY
TOPO
attB1
attB2
TOPO
pEXP-TOPO

FIG. 33

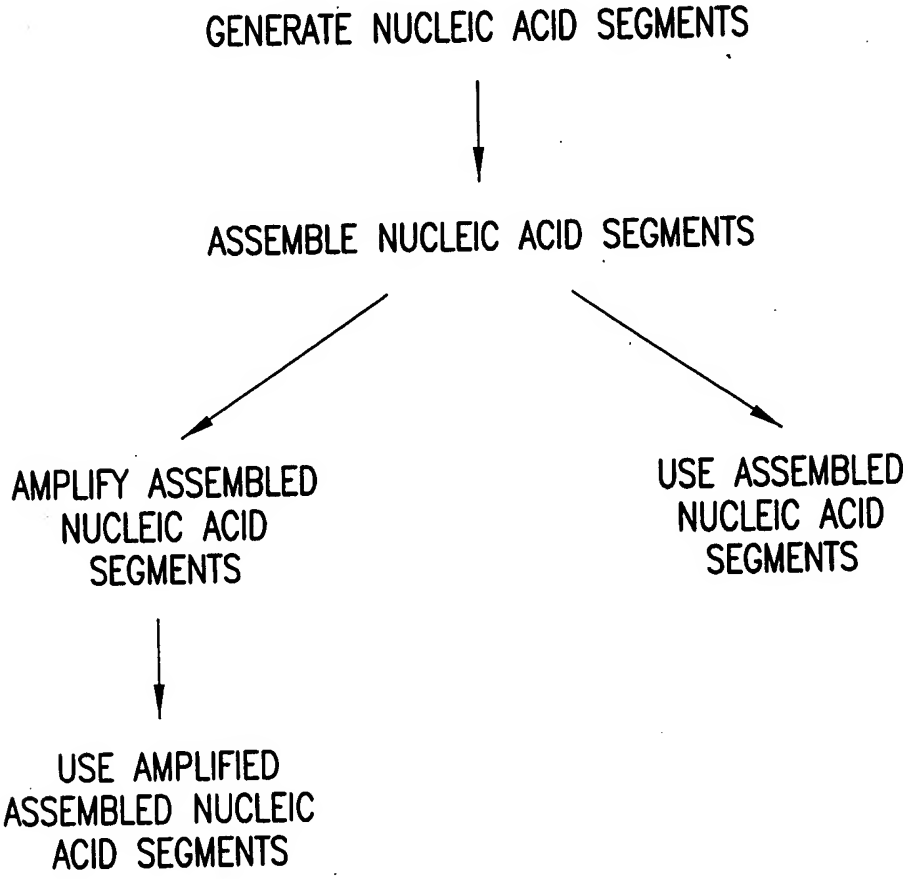

GENERATE NUCLEIC ACID SEGMENTS
ASSEMBLE NUCLEIC ACID SEGMENTS
AMPLIFY ASSEMBLED NUCLEIC ACID SEGMENTS
USE ASSEMBLED NUCLEIC ACID SEGMENTS
USE AMPLIFIED ASSEMBLED NUCLEIC ACID SEGMENTS

FIG. 34

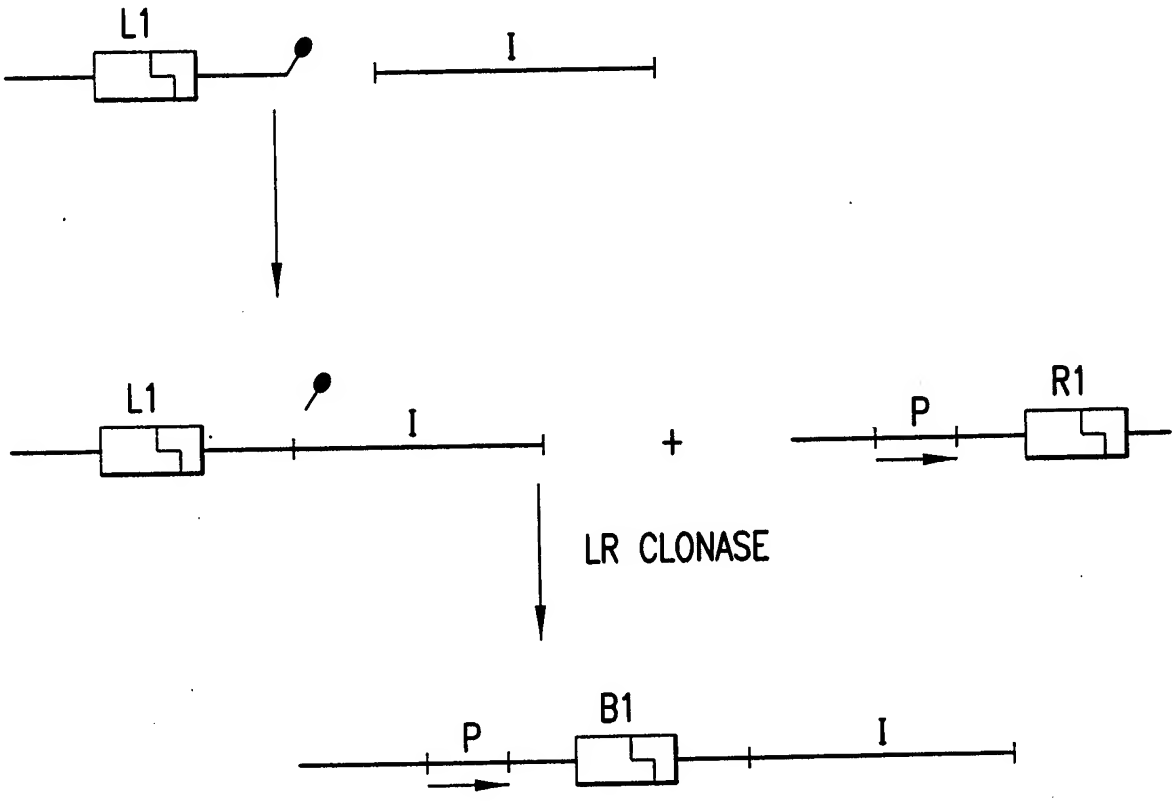


FIG. 35

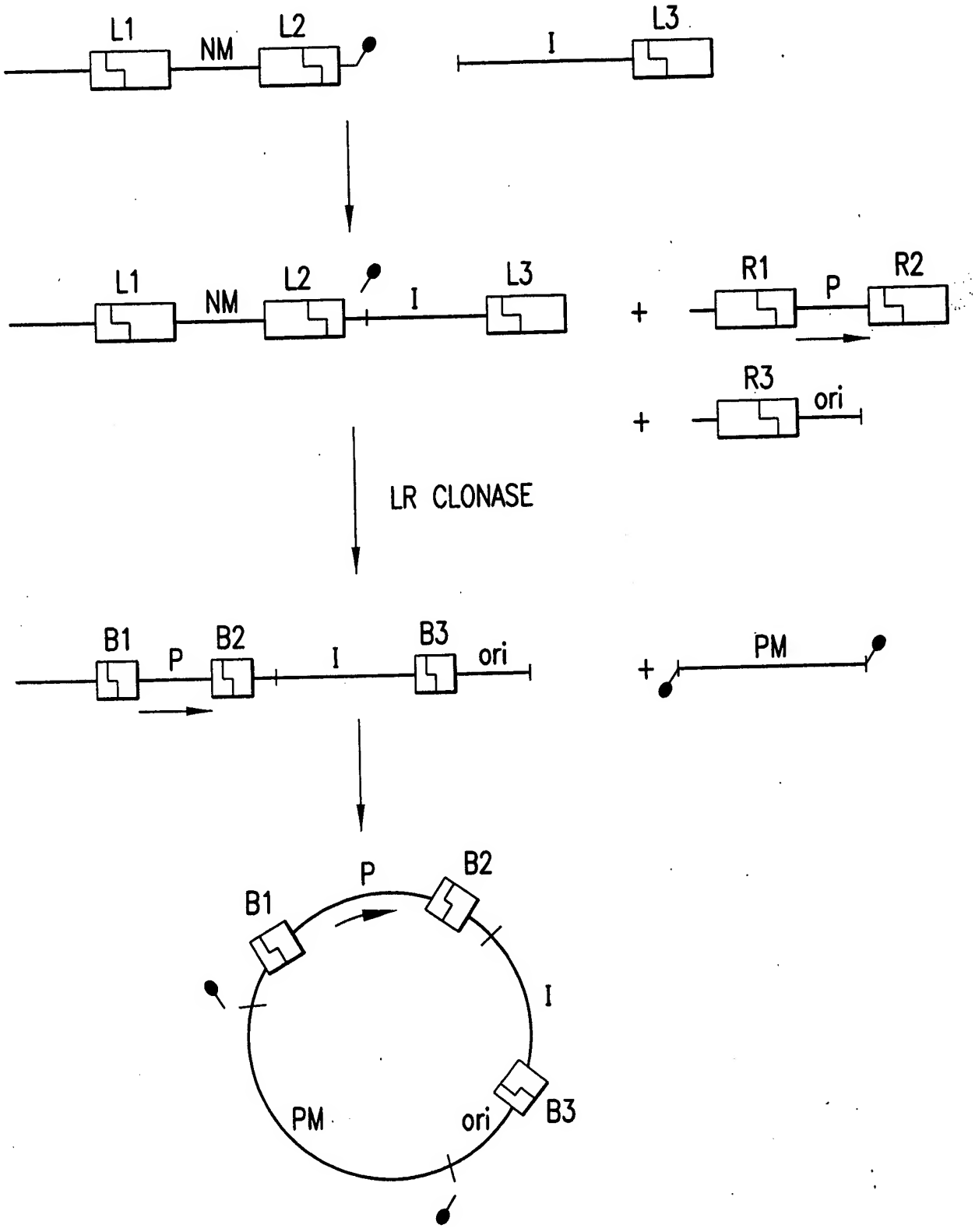

L1
NM
L2
I
L3
L1
NM
L2
I
L3
R1
P
R2
R3
ori
LR CLONASE
B1
P
B2
I
B3
ori
PM
P
B2
B1
I
PM
ori
B3

FIG. 36

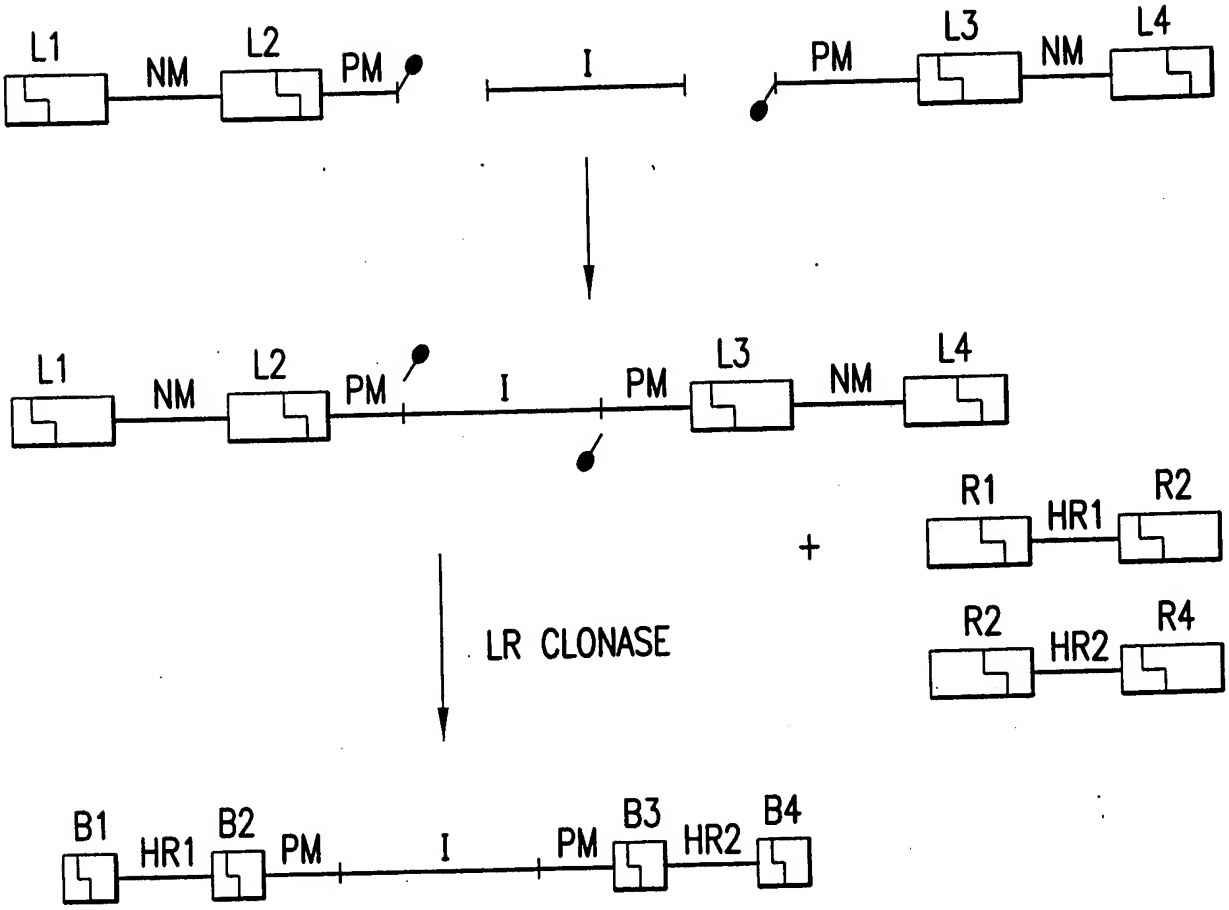

L1
NM
L2
PM
I
PM
L3
NM
L4
L1
NM
L2
PM
I
PM
L3
NM
L4
R1
HR1
R2
+
LR CLONASE
R2
HR2
R4
B1
HR1
B2
PM
I
PM
B3
HR2
B4

FIG. 37

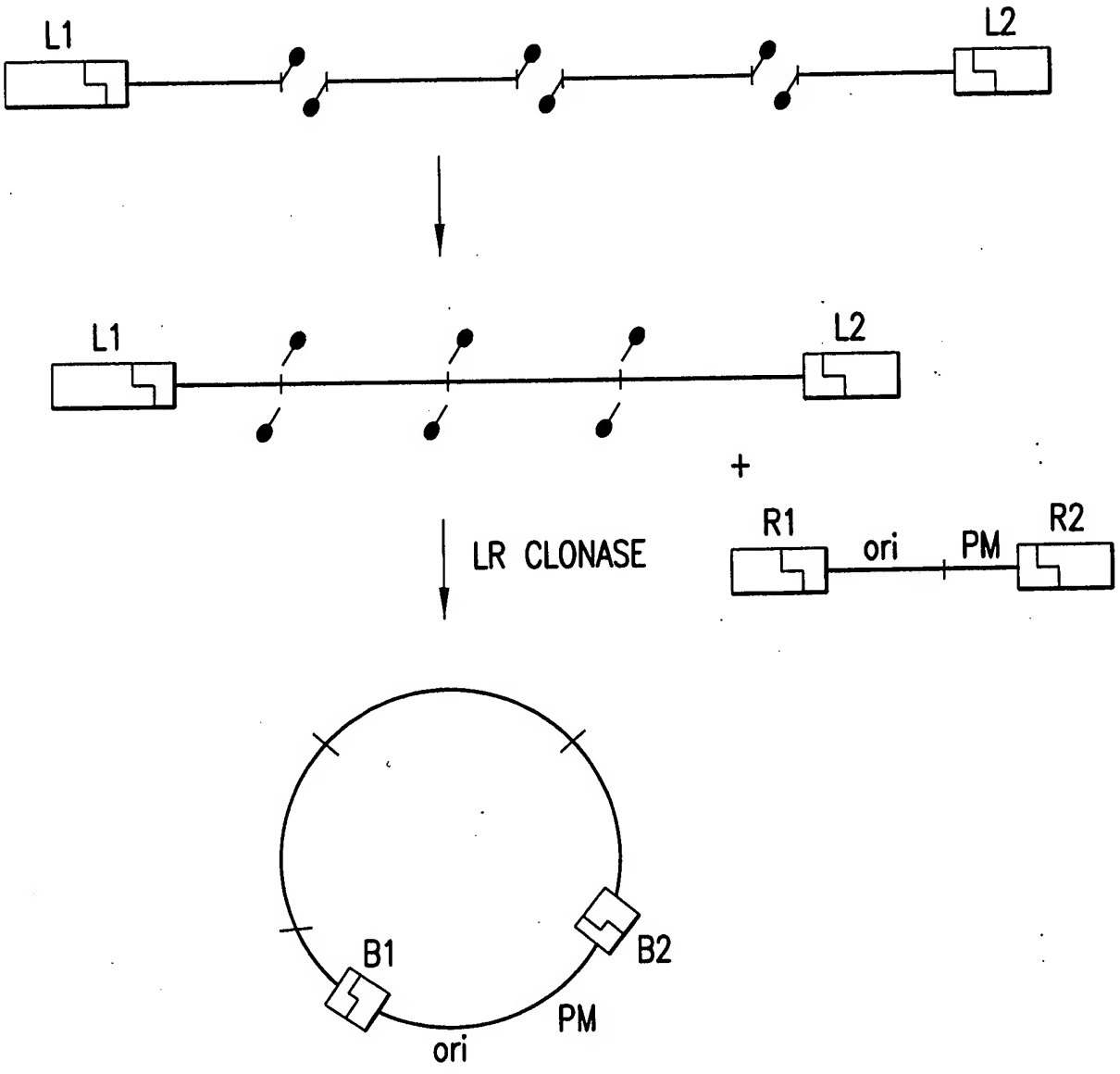


FIG. 38

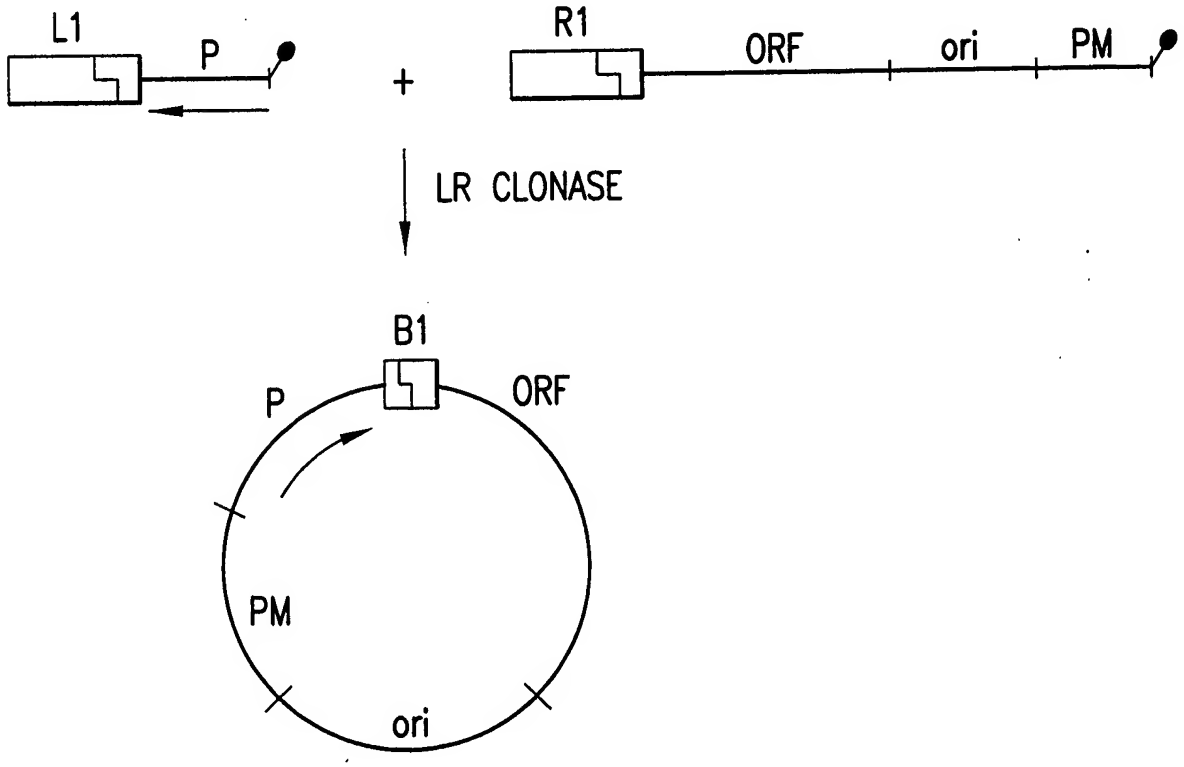


FIG. 39

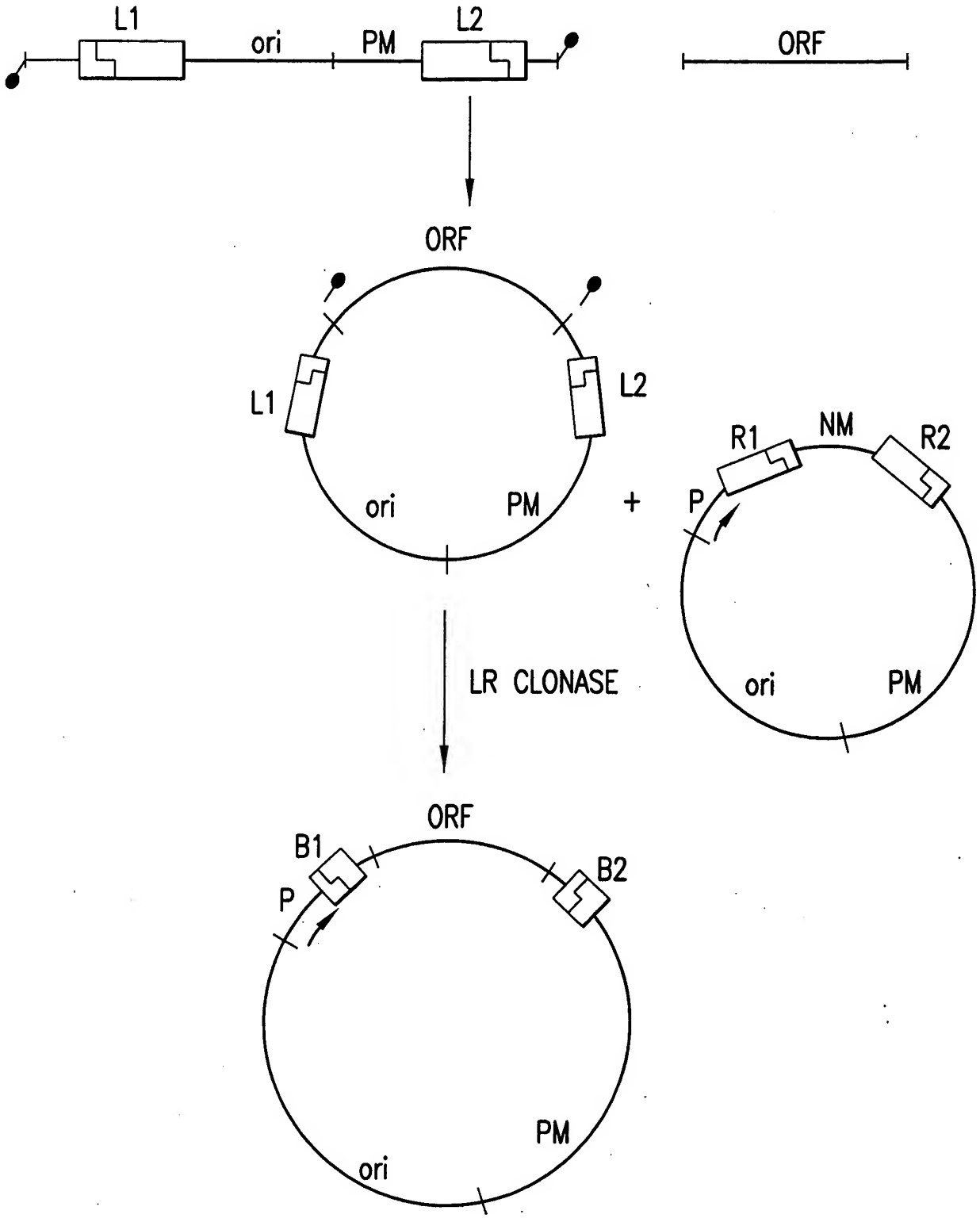

L1
ori
PM
L2
ORF
ORF
L1
L2
ori
PM
+
R1
NM
R2
P
ori
PM
LR CLONASE
ORF
B1
B2
P
PM
ori

FIG. 40